Katholieke Universiteit Leuven
Biomedical Sciences Group
Faculty of Medicine
Department of Cardiovascular Sciences
Center for Molecular and Vascular Biology

DEVELOPMENT OF PREDICTION MODELS FOR ALLOGRAFT VASCULOPATHY IN HEART TRANSPLANT RECIPIENTS

Neha SINGH

Jury:

Promoter:	Prof. Dr. B. De Geest
Co-promoter:	Prof. Dr. J. Van Cleemput
Chair:	Prof. Dr. B. Meyns
Secretary:	Prof. Dr. W. Budts
Jury members:	Prof. Dr. L. Potena Prof. Dr. G. De Meyer Prof. Dr. M. Jacquemin Prof. Dr. M. Naesens

Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in Biomedical Sciences

March 2015

Acknowledgements

First and foremost, I would like to express my deepest gratitude to my promoter, Prof. Dr. Bart De Geest, for his excellent guidance, continuous support, and motivation throughout my PhD research. I would like to thank you for encouraging my research and for allowing me to grow as a research scientist. I appreciate your comprehensive knowledge, constant feedback, and great leadership, which made my PhD experience productive and efficient. It is truly an honor to have the wonderful opportunity to successfully complete my PhD under your supervision.

I express my sincere gratitude to my co-promoter Prof. Dr. Johan Van Cleemput. Your striking observations and valuable inputs have sent my research in the right direction. I am extremely grateful for your unconditional help, moral support, and consistent encouragement that I received throughout my PhD. Under your supervision, I got the opportunity to learn a lot and overcome many difficulties during these years. Thanks for the patience and time you invested in me with guidance and scientific discussions.

My sincere thanks also goes to Prof. Dr. Johan Vanhaecke who offered his strategic insights and guidance throughout the project. I am grateful to you for showing interest in my work and being very encouraging and supportive.

I would also like to thank Prof. Dr. Marc Jacquemin, Prof. Dr. Werner Budts and Prof. Dr. M. Naesens for their valuable suggestions and insightful comments. I am grateful to Prof. Dr. Guido De Meyer and Prof. Dr. Luciano Potena for serving as my PhD examination committee external members and for their evaluation of the manuscript. My sincere thanks to Prof. Dr. Bart Meyns for his commitment as chairman of the jury.

I thank Prof. Dr. Marc Hoylaerts, Dr. Jan Emmerechts, Dr. Marc Tjwa, Dr. Vincent Carlier, Dr. Kris Covens and Dr. Ward Heggermont for the constructive research collaborations, good advice, and scientific contributions to this work. I am grateful to Dr. Steffen Fieuws for the valuable help in the statistical analyses of data and for sharing his critical knowledge on statistics and on prediction models.

My profound gratitude goes to Prof. Dr. E. Verbeken for generously providing magnificent images of histological sections illustrating human cardiac allograft vasculopathy. The image on the cover of this PhD thesis is a courtesy of Prof. Dr. E. Verbeken.

I would especially like to thank Kelly Buekenhoudt, Nathalie Fransen, Sabine Gryp, Dominica Kums, all the nurses, and nurse aids in the heart transplantation unit at University Hospital Leuven. All of you have been there to support me when I recruited patients and collected data for my PhD thesis. Thank you all for being kind enough and extending your help at various phases of this research.

I would like to share the credit of my work with my past and present colleagues Dr. Frank Jacobs, Dr. Eline Van Craeyveld, Dr. Stephanie Gordts, Ilayaraja Muthuramu and Elena Nefyodova who accompanied me during various periods of my PhD. I thank you all for providing a great atmosphere at our office, for the constructive discussions, and for sharing your knowledge with me. I am grateful to all of you for your help in many different ways including finding a living place when I arrived in Belgium. Thanks for making me familiar with the Belgian culture and the best Belgian chocolates. I truly admire the friendship and support I received from all of you in these years. It was a pleasure to work alongside the current group members Ruhul and Rayhanul. I wish them success in their future endeavors.

I thank Diane De Wyngaert, Gabriela Casteels, Karen Cauwels, Dr. Bianca Hemmeryckx, Dr. Benedetta Izzi, Christine Vranckx, Chantal Thys, Petra Vandervoort, Boukje Hoekman, Soetkin Van Kerckhoven, Renaud Lavend' Homme, Paolo Carai, and all the colleagues from CMVB whose help I have appreciated over the course of this PhD. It was a great experience working alongside with you in all these years.

I would like to thank my close friend Vinay for his unwavering support, kindness, patience, and valued advice throughout these years. Without your help, encouragement and faith in me, this thesis would not have been possible.

Thanks to my best friends Neha and Chhavi for their persistent support and motivation. I would also like to thank Naba, Bahar, and all of my friends who supported and motivated me to strive towards my goal and made my time enjoyable at KU Leuven.

A special thanks to my family for instilling in me confidence and a drive for pursuing my PhD. Words cannot express how grateful I am to my family for the constant encouragement and support. It was your motivation and blessings for me that sustained me on the right path.

Neha
March 2015

Table of contents

List of abbreviations

ABCA1	ATP-binding cassette transporter A1
Apo	apolipoprotein
AUC	area under the curve
BFU-E	erythroid burst-forming units
BOECs	blood outgrowth endothelial cells
CAV	cardiac allograft vasculopathy
CAD	coronary artery disease
CEC	circulating endothelial cell
CEMP	circulating endothelial microparticle
CFU-E	erythroid colony-forming units
CFU-G	granulocyte colony-forming units
CFU-GEMM	granulocyte, erythrocyte, macrophage, megakaryocyte colony-forming units
CFU-GM	granulocyte, macrophage colony-forming units
CFU-M	macrophage colony-forming units
CMV	cytomegalovirus
CPMP	circulating platelet microparticle
CXCL12	C-X-C motif chemokine 12
CRP	C-reactive protein
DAPI	4’,6-diamidine-2-phenylidole dihydrochloride
DiI-acLDL	1,1’-dioctadecyl-3,3,3’,3’- tetramethylindocarbocyanine-labeled acetylated LDL
ECFCs	endothelial colony forming cells
ELISA	enzyme-linked immunosorbent assay
EPC	endothelial progenitor cell
HDL	high-density lipoprotein
HLA	human leukocyte antigen
HPC	hematopoietic progenitor cell
IQR	interquartile range

IL	interleukin
IVUS	intravascular ultrasound
LDL	low-density lipoprotein
MHC	major histocompatibility complex
miRNA	microRNA
MIT	maximal intimal thickness
MNC	mononuclear cells
mTOR	mammalian target of rapamycin
NF-AT	nuclear factor of activated T cells
OR	odds ratio
SEM	standard error of the mean
SDF-1α	stromal derived factor-1α
UEA-I	*Ulex europaeus* agglutinin-I
VEGFR-2	vascular endothelial growth factor receptor-2

Chapter 1

General Introduction

1.1 HEART TRANSPLANTATION: A HISTORICAL PERSPECTIVE

The first human-to-human heart transplant was performed by Christiaan Barnard in South Africa on December 3 1967[1]. Pioneering experimental work had been performed by Alexis Carrel, Charles Claude Guthrie, Frank Mann, Norman Shumway, and Richard Lower[2]. Alexis Carrel received the 1912 Nobel Prize in Medicine or Physiology for the development of the technique for suturing blood vessels[3]. Seminal experiments in organ transplantation were conducted by Alexis Carrel and Charles Claude Guthrie at the University of Chicago in 1904 and 1905[4-6]. Together with the aviator Charles Lindbergh, Alexis Carrel invented in the 1930s a mechanical heart that circulated vital fluids through excised organs. In the 1930s, Frank Mann and colleagues were able to perform heterotopic heart transplantation with puppy hearts with survival of up to eight days[7]. In 1958 Keith Reemtsma demonstrated that immunosuppressive agents prolong heart transplant survival in the laboratory setting. At the end of the 1950s beginning of the 1960s, Norman Shumway and Richard Lower performed revolutionary experimental work in Stanford University on developing the technique of orthotopic heart transplantation in dogs. By the mid-1960s, their experience led to the conviction that immunologic rejection was the only remaining obstacle to successful clinical heart transplantation. However, human heart transplantation was also delayed in the USA due to legal restrictions on the use of brain dead donors.

The first South African heart transplant patient initially recovered well, but died of pneumonia 18 days later. The second South African heart transplant recipient was the first able to leave the hospital and to lead a relatively normal life. He survived for 19 months and died of myocardial infarction[2].

The number of heart transplants dropped from 100 in 1968 to only 18 in 1970 because of generally poor survival results. Alloimmune responses could not be tackled efficiently. Norman Shumway was the only American surgeon to continue performing heart transplantations. Results of heart transplantation improved in the second half of the 1970s but the practice was restricted to a very limited number of centers. Shumway pioneered the use of cyclosporine, originally isolated from the soil fungus Tolypocladium inflatum, in heart transplant recipients. In November 1983, cyclosporine was approved for commercial use. The introduction of this drug was pivotal for the rise of the heart transplantation field.

Orthotopic heart transplantation is now a well-established therapy for selected patients with end-stage congestive heart failure. Whereas this procedure represents one of the most remarkable breakthroughs of the past century, a variety of medical problems occur during follow-up of heart transplant recipients. Morbidities in heart transplant recipients include acute rejection episodes, opportunistic infections, solid and hematological malignancies, renal insufficiency, hypertension, osteoporosis, diabetes, and cardiac allograft vasculopathy (CAV). After the first year, CAV and late graft failure are the most important cause of death (approximately 30% of mortality) in heart transplant recipients followed by malignancy (20%), non-cytomegalovirus (CMV) infection (12%), and renal failure (8%)[8-11]. Severe CAV represents an important indication for re-transplantation. CAV can be considered to be a manifestation of chronic rejection. In contrast to liver, lung, and

kidney transplants, diagnosis of chronic rejection in cardiac allografts is not based on biopsies but on coronary angiography and/or intravascular ultrasound.

Most of the survival improvement in heart transplant recipients in the past three decades is related to mortality reduction during the first post-transplant year[10]. Mortality beyond 1 year after transplant has only slightly declined for patients transplanted in the past 20 years. This lack of progress may partially be related to a donor and recipient pool with higher risk characteristics. Nevertheless, interventions resulting in a reduction of events leading to long-term mortality are needed to achieve further improvements in survival after heart transplant.

1.2 PATHOLOGY OF CARDIAC ALLOGRAFT VASCULOPATHY

Severe narrowing of epicardial and smaller intramyocardial arteries is the most important determinant of cardiac allograft survival and a major cause of death in heart transplant recipients[8-10,12-14]. CAV is a generic name for the pathological abnormalities in arteries and veins of transplanted hearts. Several alternative terms have been used to describe cardiac allograft vasculopathy: transplant coronary artery disease, allograft arteriopathy, cardiac transplant arteriosclerosis, transplant atherosclerosis, and transplant vasculopathy. With the exception of 'transplant vasculopathy', all other terms tend to be restrictive or even incorrect. However, these various terms partially reflect the heterogeneity of lesions in cardiac allografts[14-18]. The lesions of CAV can be globally categorized into three different entities: atherosclerosis, intimal fibromuscular hyperplasia, and vasculitis.

Atherosclerosis in epicardial arteries is characterized by the presence of atheromata that contain a lipid core filled with extracellular cholesterol and cellular debris and are covered by a fibrous cap. Inflammation is most often limited to the intima. Atherosclerotic plaques generally occur proximally, spare the intramyocardial arteries, and tend to be eccentric.

In contrast to atherosclerosis, fibromuscular intimal hyperplasia does not contain atheromata, but mainly consists of smooth muscle cells and extracellular matrix. Nevertheless, intracellular lipid containing foam cells may be present in fibromuscular intimal hyperplasia. Fibromuscular intimal hyperplasia is the most prominent lesion of CAV and lesions tend to be circumferential. It not only involves the large epicardial arteries but also smaller epicardial arteries and intramyocardial arteries. It may also occur in veins. Whereas calcification is an important hallmark of atherosclerosis, it is usually not present in fibromuscular intimal hyperplasia.

Vasculitis is characterized by pronounced inflammation. This inflammation is frequently transmural involving intima, media, and adventitia. It occurs in arteries and veins and may be associated with destruction of the internal elastic lamina and with necrosis and/or fibrosis of the media. Vasculitis occurs in regions of fibromuscular intimal hyperplasia but there appears to be no correlation between the degree of inflammation and the proliferative component of the lesion[18]. Infiltrating cells are CD3-positive T lymphocytes and CD68-positive macrophages[18]. Inflammatory lesions may be restricted to 'endothelitis' with subendothelial accumulation of inflammatory cells, predominantly T cells.

The presence of atherosclerosis in allografts has often been attributed to pre-existing lesions in the donor. Whereas this may be correct to some degree in adult heart transplant recipients, transplant atherosclerosis is also present in pediatric heart transplant recipients with a young donor heart[18]. Therefore, the kinetics of development of atherosclerosis in transplant recipients is significantly faster than in native atherosclerosis.

In conclusion, CAV can be summarized as the triad fibromuscular intimal hyperplasia, atherosclerosis, and vasculitis. A further component of vascular pathology in allografts is microvasculopathy. In 2007, Hiemann *et al*.[19] concluded that histologically proven stenotic microvasculopathy involving the media was a novel prognostic factor for long-term survival. The authors conducted a detailed retrospective analysis of more than 9700 endomyocardial biopsies obtained during the first year after transplantation from 873 heart transplant recipients. Significant epicardial disease (>75% luminal stenosis) was found in 19% of patients at 1 year after transplantation, whereas microvascular stenosis was observed in 43% of patients, involving predominantly the media (91%) rather than the endothelium. Neither endothelial disease nor nonstenotic disease of the media was associated with a worse prognosis. However, stenotic microvasculopathy of the media was associated with a significant reduction in overall survival and freedom from fatal cardiac events, which was independent of the presence of significant epicardial disease. Since this microvasculopathy predominantly involves the media and since atherosclerosis and fibromuscular intimal hyperplasia are intimal diseases, it is difficult to envisage that there is a close biological link between CAV and microvasculopathy. Furthermore, because of the small size of endomyocardial biopsies, the true sensitivity of this technique to detect microvasculopathy is unknown unless microvasculopathy is generalized.

1.3 CLINICAL MANIFESTATIONS AND DIAGNOSIS OF CAV

According to data of the International Society for Heart and Lung transplantation[10], the prevalence of CAV (defined as any degree of angiographically visible coronary artery disease) is 42% at 7 years. The prevalence of CAV in UZ Leuven heart transplant recipients is 12%, 25%, 38%, and 48% at 1 year, 4 years, 7 years, and 10 years after transplantation, respectively (J. Van Cleemput and J. Vanhaecke, unpublished results).

In early and intermediate stages of the disease, CAV is clinically silent. By the time CAV manifests as ischemia on functional studies, the disease process is usually well advanced[20-23]. Often, the first symptoms are serious arrhythmias, heart failure, or sudden death. Interventional treatment options are limited due to the diffuse nature of the disease, which precludes the routine use of percutaneous interventions and coronary artery bypass grafting. Therefore, identification of asymptomatic patients at early stages of CAV is critical for the design and implementation of strategies for prevention of irreversible detrimental effects of CAV on graft and patient survival.

Surveillance coronary angiogram is utilized for disease monitoring. Consequently, the end-point is a relative change of the lumen diameter compared to a reference segment of the vessel with a supposedly normal caliber. Since intimal proliferation in CAV is circumferential and since the disease process is diffuse extending into intramyocardial vessels, coronary angiography cannot

detect early CAV. In contrast, intravascular ultrasound (IVUS) is a particularly sensitive method to detect early CAV and may demonstrate significant intimal thickening in the presence of a normal coronary angiogram[24]. The validity of the increase in maximal intimal thickness quantified by IVUS as a surrogate imaging end-point for subsequent angiographic disease and for hard clinical end-points has been demonstrated[24-26]. The increase in maximal intimal thickness in any matched slice after one year predicts all-cause mortality, death or graft loss or major adverse cardiac events, and angiographic CAV[24-26]. However, serial IVUS measurements in the first year are too cumbersome and too expensive for routine clinical use.

1.4 RISK FACTORS FOR CAV

Donor risk factors for CAV are donor history of hypertension, donor age, donor diabetes, and donor tobacco use[27]. Donor age is a consistent risk factor for CAV in several studies[10,25,28,29]. Recipient risk factors are recurrent acute cellular rejection[30], CMV infection[10,26,31], dyslipidemia, hypertension, pre-existing diabetes, and new-onset diabetes[32]. The expected correlation between the number of human leukocyte antigens (HLA) mismatched between donor and recipient and the number or severity of rejection episodes and the development of CAV has not been very consistently observed[33]. Damage to conduit arteries may occur by the large catecholamine surge that often accompanies brain death in the donor[33]. This surge of catecholamines also induces contraction band necrosis in the myocardium. The presence of contraction band necrosis on endomyocardial biopsies is a risk factor for subsequent development of CAV[34].

In a cross-sectional study, Escobar *et al*.[28] demonstrated that total cholesterol, low density lipoprotein cholesterol, triglyceride levels, obesity indices, donor age, and years following cardiac transplantation ($p<0.01$) were independent predictors of the severity of intimal thickening. However, statins have become a routine therapy in heart transplant recipients since two trials demonstrated the efficacy of pravastatin and simvastatin for the prevention of CAV[35,36]. The only lipid parameter that is quite consistently and independently associated with CAV in the statin era is the triglyceride level[25,29,37]. These observations may be linked with data of a study of Valantine *et al*.[38]. This report showed that high glucose or insulin plasma concentrations are predictive of intimal thickness[38]. Furthermore, weight indices are also a predictor of CAV[25,28,37]. Taken together, the aggregate information on these metabolic parameters suggests that insulin resistance may play a role in the development of CAV[38].

A multivariable analysis of the Registry of the International Society for Heart and Lung transplantation[10] identified the following risk factors: increasing donor age ($p<0.0001$), azathioprine versus mycophenolate mofetil (RR 1.56; $p<0.0001$), cyclosporine versus tacrolimus (RR 1.30; $p<0.0001$), and acute rejection prior to discharge (RR 1.30; $p<0.0001$)[10]. Tacrolimus and mycophenolate mofetil with or without corticosteroids are currently the standard maintenance immunosuppressive therapy in most centres.

Since CAV and late graft loss secondary to CAV are the most important cause of death after the first year, the question arises whether the current, quite disparate knowledge on risk factors for

CAV is sufficient to take pre-emptive measures and to develop new strategies to counteract CAV. The development of risk prediction models will be discussed in paragraph 1.15.

1.5 MAJOR HISTOCOMPATIBILITY ANTIGENS: ROLE IN ADAPTIVE IMMUNE RESPONSES

In humans, major histocompatibility (MHC) antigens are named human leukocyte antigens (HLA). Class I HLA molecules (HLA A, B, and C) are expressed on all nucleated cells. They present endogenous peptides from within the cell itself and interact with $CD8^+$ T cells. The expression of class II HLA molecules (HLA DR, DP, DQ) is restricted to antigen presenting cells (dendritic cells, macrophages, B cells) but can also be induced on other cells (e.g. endothelial cells) in the context of inflammation. Class II molecules bind peptides that have been derived from the environment and engage $CD4^+$ T cells.

Activation of T cells not only requires recognition of MHC by the T cell receptor (signal 1) but also costimulation (signal 2). The best characterised costimulatory molecules are CD28 and CD154 on T cells and their corresponding ligands CD80/CD86 (B7-1/B7-2) and CD40, respectively, on antigen presenting cells. If T cells receive both signals, an increase of intracellular calcium occurs and induces activation of the calcium/calmodulin sensitive molecule, calcineurin. Activated calcineurin induces a dephosphorylation of nuclear factor of activated T cells (NF-AT) leading to the translocation of this transcription factor to the nucleus. This results in increased transcription and translation of interleukin (IL)-2. IL-2 binds to its receptor on the cell surface. The IL-2 receptor comprised three subunits. The α subunit of the IL-2 receptor is CD25. Signaling through CD25 initiates a kinase-dependent cascade that is partially mediated through the mammalian target of rapamycin (mTOR). This signaling cascade leads to progression of the cell cycle from G1 to S and T cell proliferation ensues.

1.6 IMMUNOSUPPRESSIVE DRUGS IN HEART TRANSPLANTATION

One of the major differences between experimental animal studies investigating allograft vasculoapthy and clinical CAV in humans is that human heart transplant recipients are treated with immunosuppressive drugs. Besides corticosteroids, three main classes of immunosuppressive medications can be discerned: calcineurin inhibitors, purine synthesis inhibitors, and proliferation signal inhibitors.

As described above, transcription of IL-2 is induced following activation of the T cell receptor, calcium release, and calcium/calmodulin induced activation of calcineurin. Cyclosporin (Cyclosporin A) and tacrolimus (FK506) are calcineurin inhibitors. Cyclosporin binds to the cytosolic protein cyclophilin (immunophilin) of T lymphocytes. The complex of cyclosporin and cyclophilin inhibits calcineurin. Tacrolimus binds to the 12-kDa immunophilin FK506 binding protein (FKBP12). The FKBP12-FK506 complex interacts with and inhibits calcineurin. Important side effects of cyclosporin are gingival hyperplasia, hypertension, hypercholesterolemia, and nephrotoxicity. Tacrolimus has a less pronounced effect on blood pressure, on serum cholesterol,

and on the gingiva. However, diabetes, neurotoxicity, and diarrhea occur more often than in patients taking cyclosporin. Results of randomized trials have demonstrated that tacrolimus was superior compared to cyclosporin in the prevention of acute rejection episodes[39,40]. CAV analysed by coronary angiography was similar between cyclosporin- and tacrolimus-treated patients in a single center randomized trial[41]. However, follow-up in this trial was limited to 5 years, which may be too short for angiographic CAV as an end-point.

Azathioprine is metabolized into the active 6-mercaptopurine, which is an inhibitor of purine synthesis. Suppression of DNA synthesis by 6-mercaptopurine inhibits the proliferation of T cells and B cells. Mycophenolate mofetil acts as a reversible inhibitor of inosine monophosphate dehydrogenase in purine biosynthesis. Consequently, it inhibits lymphocyte proliferation. Other cells are able to recover purines via a separate pathway and are therefore able to escape the effect of mycophenolate mofetil. Compared to azathioprine, mycophenolate mofetil is associated with less bone marrow suppression and fewer opportunistic infections. A randomized trial demonstrated that the combination of mycophenolate mofetil and cyclosporin was associated with a 35% reduction in 3-year mortality or graft loss compared to the combination of azathioprine and cyclosporin[42]. Analysis of the development of CAV by intravascular ultrasound showed reduced progression in the mycophenolate mofetil group[43]. In an observational study of Kaczmarek *et al*.[44], the rate of freedom from CAV at 5 years was 47% with cyclosporin/azathioprine, 66% with cyclosporin/mycophenolate mofetil, 60% with tacrolimus/azathioprine and 70% with tacrolimus/mycophenolate mofetil. Multivariate Cox regression analysis revealed that mycophenolate mofetil was associated with a lower incidence of CAV ($p = 0.041$)[44].

Sirolimus, also known as rapamycin, belongs to the proliferation signal inhibitors. It binds to the immunophilin FKBP12 similar as tacrolimus. In contrast to the tacrolimus-FKBP12 complex, the sirolimus-FKBP12 complex does not inhibit calcineurin but binds mTOR Complex 1 (mTORC1). Consequently, IL-2 signaling is suppressed. Everolimus is a derivative of sirolimus and is also an mTOR inhibitor. Important side-effects of everolimus are impaired wound healing, significant nephrotoxicity when used in combination with standard doses of calcineurin inhibitors, and mouth ulcers[45]. In a randomized clinical trial comparing everolimus and azathioprine, everolimus significantly reduced the proportion of patients reaching at 6 months the primary efficacy end-point composed of death, graft loss or re-transplantation, loss to follow-up, biopsy-proved acute rejection of grade 3A, or rejection with hemodynamic compromise[46]. Everolimus was more efficacious than azathioprine in reducing the severity and incidence of cardiac-allograft vasculopathy at 12 months[46]. In a randomized trial comparing everolimus plus reduced dose calcineurin inhibition versus standard dose calcineurin inhibition, CAV progression was attenuated with everolimus versus standard calcineurin inhibition when patients on azathioprine therapy were considered[47]. However, in patients receiving mycophenolate mofetil, accelerated CAV progression occurred with everolimus versus standard calcineurin inhibition. Beneficial effects of sirolimus on progression of CAV have also been demonstrated[48,49].

An important caveat is that trials on the effect of mycophenolate mofetil and of mammalian target of rapamycin inhibitors inhibitors were conducted on a background of cyclosporine and steroids

and comparison of the drug of interest (mycophenolate mofetil, everolimus, sirolimus) was made with azathioprine. Standard maintenance immunosuppressive therapy in most centres consists at present of the calcineurin inhibitor tacrolimus combined with mycophenolate mofetil with or without steroids. This implies that the question whether mammalian target of rapamycin inhibitors (everolimus, sirolimus) are superior compared to mycophenolate mofetil in prevention of CAV cannot be answered.

1.7 ALLOIMMUNE RESPONSES AND MHC RESTRICTION: A CHALLENGING PARADOX IN CELLULAR IMMUNITY

Incompatibility between donor cells and recipient cells is the main threat for the long-term success of solid organ transplantation. Allorecognition involves the detection of non-self antigens by the host immune system[50]. The recognition of self versus non-self occurs via MHC. The number of HLA polymorphism in humans is very high. There is possibility for variation in all twelve classic HLA alleles. Intact MHC molecules are a major target in allorecognition. There are also minor histocompatibility antigens e.g. the Y-linked minor histocompatibility antigens (H-Y antigen)[51].

T cell alloreactivity, involving recognition of allogeneic MHC molecules by MHC-restricted T cells, is a challenging paradox in cellular immunity[52]. The primary function of MHC molecules is to initiate an immune response by presenting antigenic peptides on the cell surface for recognition by the T cell receptor[52]. As demonstrated by Zinkernagel and Doherty in 1974, this interaction is restricted: it occurs only between T cells and antigen presenting cells originating from a syngeneic background[53]. In humans, MHC restriction basically implies that T lymphocytes can only detect an antigen if this antigen is displayed by an MHC molecule from the same individual. MHC restriction is the result of positive selection in the thymus: immature thymocytes that initially express both the CD4 and CD8 co-receptors undergo apoptosis if they do not receive a positive survival signal through their T cell receptor by interaction with thymic epithelial cells that present self-peptides bound to MHC. Positive selection by weak interaction with these self-peptide:MHC complexes leads to further maturation into single positive $CD4^+$ or $CD8^+$ lymphocytes[54]. Immature thymocytes that react too strongly with self-peptide:MHC complexes undergo clonal deletion by apoptosis. This is negative selection[55]. As a result of positive and negative selection, mature lymphocytes exhibit specificity for self-MHC:foreign antigen complexes and lack autoreactive properties.

MHC restriction of T lymphocytes is the logical consequence of positive and negative selection in the thymus. The critical role of the displaying MHC molecules in antigen presentation reflects that the T cell receptor recognizes some residues of an antigenic peptide together with some residues from the displaying MHC molecule. Based on the principle of MHC restriction, it seems contradictory that T cells may react against a foreign peptide:foreign MHC complex. This appears to be due to the fact the MHC molecule of the donor is similar to self-MHC molecule in the binding region to the T cell receptor. Consequently, the foreign peptide:foreign MHC complex is recognized as an apparent foreign peptide:self-MHC complex.

1.8 ALLOIMMUNE RESPONSES: THE DIRECT AND THE INDIRECT PATHWAY

There are two pathways of T cell activation in alloimmunity. The direct pathway is unique to transplantation. Recipient $CD4^+$ and $CD8^+$ T cells recognize and respond to donor peptide:donor HLA complexes on graft-derived cells. Specifically, $CD8^+$ T cells directly engage endogenous donor peptide:HLA class I complexes on donor APCs, receive costimulatory signals, and become activated. $CD4^+$ T cells may be directly activated by donor peptide:MHC class II complexes on donor APCs. Since T cell activation requires costimulatory signals, the direct pathway will be highly dependent on bone marrow-derived, specialized APCs from the donor. Once the donor APCs have been depleted from the donor heart, as occurs within weeks of transplantation, the parenchymal and resident cells of the donor heart are incapable of driving direct pathway activation of recipient T cells. On the contrary, direct allorecognition in the absence of costimulation may have a tolerizing effect[56]. However, donor endothelial cells may also play a role in direct alloantigen presentation (*cfr. infra*).

Indirect allorecognition occurs when donor histocompatibility molecules are internalized, processed, and presented as peptides by recipient antigen presenting cells. Thus, the fundamental difference between the direct and indirect pathway is that in the former the antigen presenting cell is of donor origin. In the indirect pathway, host T cells recognize graft alloantigens presented by a host APC. This form of antigen presentation by the MHC class II molecule targets class II-restricted host $CD4^+$ T cells, resulting in their activation in response to the graft. The direct pathway likely plays a dominant role in acute rejection. The indirect pathway is likely to be permanently active since recipient APCs migrate through the graft and may pick up donor antigens. The indirect pathway plays a role in acute rejection episodes and in chronic rejection. Chronic rejection is the likely key player of the development of CAV. Chronic rejection also occurs in kidney allografts, lung allografts, and liver allografts and the corresponding pathologies are named chronic allograft nephropathy, bronchiolitis obliterans and vanishing bile-duct syndrome.

1.9 THE ROLE OF THE ENDOTHELIUM IN ALLOIMMUNITY

Endothelial cells of coronary arteries are the first biological interface between the donor allograft and circulating immunocompetent cells of the heart transplant recipient. The endothelium is activated in allografts. This implies expression of adhesion molecules (e.g., E-selectin, intercellular adhesion molecule-1), secretion of cytokines and chemokines, and also expression of molecules that provide costimulatory signals for lymphocyte activation. Importantly, activated $CD4^+$ T cells stimulate the expression of HLA class-II molecules on endothelial cells.

Whereas the endothelium may be a target of alloimmune responses, donor endothelial cells may initiate allospecific immune responses by expressing MHC class II antigens with subsequent direct activation of $CD4^+$ T cells[57]. It has been shown that direct $CD8^+$ T cell activation by endothelial cells can trigger acute cardiac allograft rejection[58]. Taken together, human graft endothelial cells of donor origin may activate allogeneic host T cells through direct presentation of MHC molecules in

the presence of sufficient costimulation[59]. On the other hand, it has been suggested that graft endothelium replacement by recipient-type cells is required for the rejection of cardiac allografts mediated by indirect pathway alloreactive $CD4^+$ T cells[60]. In any case, it is uncertain whether replacement of donor endothelium by recipient endothelial cells is beneficial. As long as the endothelium is activated, recipient dendritic cell invasion is increased[61]. These dendritic cells may pick up donor alloantigens, migrate to secondary lymphoid organs, and activate $CD4^+$ T cells via the indirect pathway.

1.10 THE ROLE OF THE ENDOTHELIUM IN THE PATHOGENESIS OF CAV

The endothelium regulates vascular tone, inflammation, smooth muscle cell proliferation, and thrombosis[62]. In the 'response to injury' concept of CAV, vascular lesions are considered to be the result of cumulative endothelial injury both by alloimmune responses and by non-alloimmune insults[63] (Figure 1.1). T cell alloimmunity, antibody-mediated immune attack, and non-immune factors induce endothelial cell death or endothelial dysfunction. Cellular infiltrates in the vessel wall of heart transplant recipients with CAV are predominantly T cells that are mainly localized in the neointima and the adventitia[64]. Endothelitis associated with subendothelial accumulation of T lymphocytes is a manifestation of chronic rejection. Endothelial injury initiates an inflammatory cascade, which is associated with the activation of macrophages and proliferation of smooth muscle cells. Recruitment of monocytes is dependent on chemokines and cytokines produced by T cells and endothelial cells. Concentric intimal thickening is the outcome of smooth muscle cell migration and proliferation as a result of the dysfunctional endothelium and cytokines produced by monocytes. The process of endothelial injury is counteracted by endothelial repair mechanisms that will be discussed in detail below.

Endothelial injury likely plays a central role in the pathogenesis of CAV. However, the scheme of the pathogenesis of CAV described above is highly conceptual and does not make a distinction between true causes (*conditio sine qua non*) and modifiers. Since CAV is only observed in transplant patients and specifically in allografts and not in isografts, alloimmunity plays an overwhelming and causative role in the development of CAV. CAV has long been referred to as chronic rejection[8]. Non-alloimmune factors are modifiers of the pathology. Endothelial injury is a mediator. It is an intermediate variable between cause or modifiers and the pathological manifestations.

One should also keep in mind that CAV is not a uniform pathological process. Although fibromuscular intimal hyperplasia is the dominant pathological manifestation, transplant atherosclerosis in large epicardial arteries is also observed. Different pathological manifestations indicate different pathogenetic mechanisms.

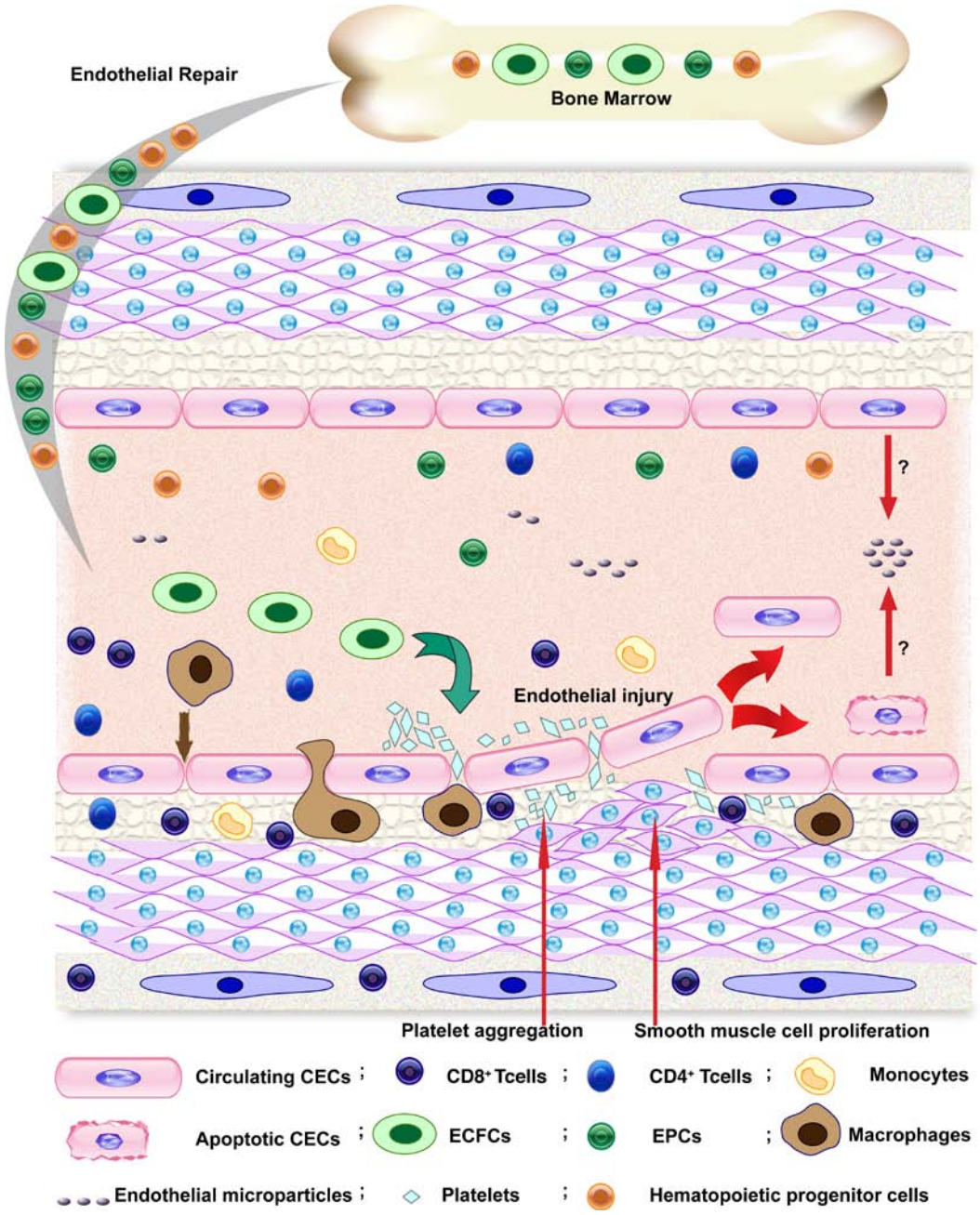

Figure 1.1. Schematic representation of the role of endothelial injury and endothelial repair in the development of cardiac allograft vasculopathy (CAV). Alloimmune endothelial injury likely plays a causal role in the development of CAV. Non-alloimmune factors may modify endothelial injury. Endothelial dysfunction and injury may initiate a cascade of events including platelet activation and inflammation with infiltration of predominantly macrophages and T cells in the intima and the adventitia. Endothelial dysfunction and injury and subsequent vessel wall inflammation induce smooth muscle cell activation, migration, and proliferation. Activation and injury of endothelial cells may also cause the shedding off of endothelial cells in peripheral blood as viable circulating endothelial cells (CECs) or as apoptotic CECs. Endothelial microparticles are also present in the peripheral blood but the exact mechanism and origin of their formation is not known. Bone marrow derived progenitor cells contribute to restoration of vascular endothelial integrity via paracrine effects or via incorporation into the endothelium . Progenitor cells include early endothelial progenitor cells (EPCs), endothelial colony forming cells (ECFCs), and hematopoietic progenitor cells (HPCs). Early EPCs and HPCs release pro-angiogenic factors whereas ECFCs directly contribute to reendothelialization.

1.11 ENDOTHELIAL REPAIR MECHANISMS

Endothelial progenitor cells (EPCs) play a role in maintaining the integrity of the vascular endothelium (Figure 1.1). In the literally correct sense of the word, EPCs are immature precursor cells capable of differentiating into mature endothelial cells *in vivo*. However, the term endothelial progenitor cell (EPC) is used in a very broad sense in the literature. EPCs encompass different categories of cells with different phenotypic and functional properties that affect neovascularization and reendothelialization. Different protocols for short-term culture of blood mononuclear cells on fibronectin (with or without gelatin)-coated plates have been established by Vasa *et al*.[65] and Hill *et al*.[66]. These cells are stimulated by culture conditions to mimic many features of endothelial cells[67]. However, the putative EPCs identified by these assays do not give rise to a lineage of endothelial progeny, but cultured cells consist of monocytes or a population of hematopoietic cells with monocyte-macrophage or T cell lineage commitment. The cells identified in these assays may regulate the angiogenic response in a paracrine way[68] by releasing angiogenic factors that promote reendothelialisation indirectly. These cells have been called circulating angiogenic cells or pro-angiogenic progenitor cells[67]. Nevertheless, EPCs defined in the literally correct sense of the word do exist. Using high-power laser multichannel scanning confocal microscopy to visualize three-dimensional vascular structures, incorporating bone marrow-derived EPCs have been unambiguously identified in allografts[69]. Incorporation of EPCs has also been observed in a murine model of vein graft atherosclerosis[70]. *In vitro*, endothelial colony forming cells (ECFCs) or blood outgrowth endothelial cells (BOECs) are derived from long-term culture of adherent peripheral blood mononuclear cells in endothelial conditions and consist of colonies that display a cobblestone morphology[70,71]. ECFCs express cell surface antigens like primary endothelium, clonally propagate, can be replated into secondary and tertiary ECFCs, form capillary-like structures *in vitro*, and become endothelial cells *in vivo*[71]. The term EPC is used in this doctoral thesis in the broad sense of the word. Taken together, increased EPC number and function may enhance endothelial repair in allografts, directly via increased EPC incorporation ('building block' role) or indirectly via production of growth factors (paracrine role).

As discussed *supra*, cells that were originally identified as EPCs are in fact hematopoietic lineage cells. Cells of the hematopoietic lineage may be mobilized from the bone marrow and are entrapped in peripheral tissues, where they release angiogenic signals. Common myeloid progenitors and granulocyte-macrophage progenitors may differentiate into pro-angiogenic cells[72] and may promote neovascularization and endothelial repair. A comprehensive analysis of endothelial repair mechanisms should therefore not be restricted to EPC quantifications but also entail quantifications of hematopoietic progenitor cells.

1.12 ENDOTHELIAL CELL DEATH AND TURNOVER

In healthy individuals, the endothelial layer is renewed at a low replication rate of 0-1% per day[73]. The measurement of immunologically-defined circulating endothelial cells (CECs) is a direct method to assess vascular integrity[74]. Endothelial cells can be activated by various stimuli such as

cytokines, growth factors, infectious agents, lipoproteins, or oxidative stress. In allografts, antibody-mediated rejection or cellular rejection may induce endothelial cell activation. Prolonged activation may lead to loss of integrity of the endothelial layer and cell detachment. Detached cells may be viable, apoptotic, or necrotic[75]. Apoptotic CECs can be quantified by including Annexin V staining in the protocol. It has been shown that cells undergoing apoptosis break up the phospholipid asymmetry of their plasma membrane and expose phosphatidylserine, which is translocated to the outer layer of the membrane[76]. Annexin V preferentially binds to negatively charged phospholipids like phosphatidylserine in the presence of Ca^{2+} and shows minimal binding to phosphatidylcholine and sphingomyeline. Quantification of CECs and apoptotic CECs represents a sensitive and specific marker of endothelial damage. In contrast, soluble markers like von Willebrand factor, thrombomodulin, and E-selectin do not distinguish between endothelial cell activation and endothelial damage, and are therefore not suitable to measure endothelial turnover.
Apoptotic circulating endothelial microparticles (CEMPs) constitute another parameter to measure endothelial damage. They result from exocytic budding following endothelial cell activation and consist of phospholipids surrounding a cytoplasmic component[77]. Apoptotic CEMPs can be quantified by flow cytometry based on size, presence of an endothelial surface marker, and Annexin V staining.

1.13 RELATION BETWEEN EPCS AND CAV

In a small study comparing 8 patients with CAV and 7 patients without CAV, Simper and colleagues[78] observed that the number of EPC colony-forming units (late outgrowth EPCs) appearing over a 6-week culture period was significantly lower in patients with CAV. In contrast, no significant difference in early EPC number was observed after 4 days of culture. The authors analyzed the origin of endothelial cells in the graft in sex-mismatched cardiac transplantation recipients based on fluorescent *in situ* hybridization for the Y chromosome and immunostaining for CD31. The percentage of recipient-derived luminal endothelial cells ranged from 1% to 24% in diseased segments but was significantly lower than 1% in non-diseased segments.
Thomas *et al*.[79] compared 17 patients with CAV and 17 patients without CAV. No difference in the number of $CD34^+$, $CD34^+CD45^+$, $CD133^+$, $CD34^+CD133^+$, $CD34^+$VEGFR-2^+, $CD133^+$VEGFR-2^+, and $CD34^+CD133^+$ VEGFR-2^+ cells was observed between both groups. The fact that 7 different categories were quantified indicates that a unique immunophenotypic definition of EPCs does not exist. More recently, Schober *et al*.[80] compared 84 patients with CAV and 103 patients without CAV. The number of $CD34^+$VEGFR-2^+ cells was not significantly different in patients with CAV and without CAV. Taken together, most evidence indicates that there is no relation between the number of EPCs and the presence of CAV. However, definitions of EPCs based on immunophenotype may not be adequate. In addition, no information on EPC function in heart transplant recipients is available.

1.14 THE ORIGIN OF THE ENDOTHELIUM IN ALLOGRAFTS

The origin of the endothelium in allografts remains relatively unclear. As discussed *supra*, the data of Simper *et al*.[78] suggest that recipient-derived luminal endothelial cells constitute a minority or a small minority of the endothelium in allografts. Whereas these data are based on sex-mismatch, information may also be obtained in the case of ABO-compatible, non-identical cardiac transplantation (A receiving heart from O, B receiving heart from O, AB receiving from heart A, B, or O). O'Connell *et al*.[81] found that the endothelial cells in the graft expressed characteristics of the recipient in 30% of recipients in the first year after transplantation. In half of these hearts, reversal of the phenotype was partial, in the other half it was complete.

Koestner *et al*.[82] reported the results on an ABO mismatched heart transplantation: a 19-year old patient with blood group type O accidentally received a blood group B cardiac allograft. This patient had an Ivemark syndrome (congenital univentricular heart, situs inversus, and asplenia). The patient died 5 years after transplantation because of cardiac allograft vasculopathy. The antigenic profile of the graft endothelial cells progressively changed between 15 and 30 months from B to O. By 44 months post-transplantation, it had changed to the O type (H antigen on endothelial cells). In ABO-mismatched heart transplants in infants, no change in the antigenic profile of the graft endothelium was observed[83].

1.15 RISK PREDICTION MODELS FOR CAV

Risk prediction models are well-established for native coronary artery disease. Examples of prognostic prediction models for coronary heart disease are The Framingham risk score[84] and Systemic Coronary Risk Estimation (SCORE)[85]. At present, there are no well-established and robust prediction models for prevalent or incident CAV.

A diagnostic prediction model was developed by Mehra *et al*. in 1995[29]. In this study, a score based on donor age higher than 35 years, a first year mean biopsy score higher than 1, and hypertriglyceridemia at two levels of risk (150-250 mg/dl or higher than 250 mg/dl) predicted the probability for severe intimal hyperplasia in 101 subjects[29]. This model was subsequently validated in 37 patients. Substantial changes in heart transplantation have occurred since 1995 in particular improved immunosuppressive therapy and altered recipient and donor characteristics. The proportion of patients being bridged to transplant with mechanical circulatory support continues to increase rapidly with 36% of patients being on mechanical support at the time of transplant in 2010[10]. The median donor age has steadily increased and is now for instance more than 40 years in Europe[10].

Instead of using biochemical markers or clinical data to predict the presence or the incidence of CAV, serial first-year IVUS data have been used as a surrogate imaging end-point to predict angiographic CAV and/or clinical outcomes[24-26]. In 1999, Kapadia *et al*.[24] were the first to report the impact of rapidly progressive intimal thickening (>0.5 mm increase in intimal thickening from baseline in the first year of transplantation) in 100 transplant recipients. Patients with first-year rapidly progressive intimal thickening had more subsequent events (death, myocardial infarction,

and heart failure) compared with patients without rapidly progressive intimal thickening (25% versus 11%) during a follow-up period of 43 months. The most solid evidence for serial first-year IVUS data as a surrogate marker to predict angiographic CAV and poor outcomes after heart transplantation comes from two publications in 2005[25,26]. Kobashigawa *et al*.[25] demonstrated that progression of maximal intimal thickening ≥ 0.5 mm in the first year after transplant predicted death or graft loss (20.8% versus 5.9%; p=0.007), the composite end-point of death or graft loss or nonfatal major adverse cardiac events (45.8% versus 16.8%; p=0.003), and had more findings of newly occurring angiographic luminal irregularities (75.2% versus 32.6%; p=0.0004) during a five-year follow-up period. Tuzcu *et al*.[26] defined rapidly progressive vasculopathy as an increase of maximal intimal thickness ≥ 0.5 mm in the first year after transplant. After a median follow-up of 5.9 years, more patients with rapidly progressive vasculopathy died as compared to those without (26% versus 11%, p=0.03). The composite end-point of death and myocardial infarction also occurred more frequently among those with rapid progression than in those without it (51% versus 16%, p<0.0001). The IVUS-defined rapid progression predicted future development of angiographic CAV. Donor transmitted lesions were not predictive of outcome. Serial first-year IVUS cannot be applied in routine clinical practice.

Based on the existing literature (cfr. paragraph 1.4), it becomes clear that traditional risk factor approaches are markedly insufficient as a foundation for risk prediction models. Biomarkers that capture key processes or key players in the pathogenesis of CAV may be the cornerstone of adequate prediction models. These cardiovascular biomarkers should not simply be evaluated in isolation for their predictive abilities but on their added predictive contribution beyond existing or established predictors[86].

1.16 REFERENCES

1. Hunt, S.A. Taking heart--cardiac transplantation past, present, and future. *N Engl J Med* **355**, 231-235 (2006).
2. Ventura, H.O. ,Muhammed, K. Historical perspectives on cardiac transplantation: the past as prologue to challenges for the 21st century. *Curr Opin Cardiol* **16**, 118-123 (2001).
3. Carrel, A. ,Guthrie, C.C. Anastomosis of blood vessels by the patching method and transplantation of the kidney. 1906 [classical article]. *Yale J Biol Med* **74**, 243-247 (2001).
4. Carrel, A. ,Guthrie, C.C. Functions of a Transplanted Kidney. *Science* **22**, 473 (1905).
5. Carrell, A. ,Guthrie, C.C. Extirpation and Replantation of the Thyroid Gland with Reversal of the Circulation. *Science* **22**, 535 (1905).
6. Carrel, A. ,Guthrie, C.C. Successful Transplantation of Both Kidneys from a Dog into a Bitch with Removal of Both Normal Kidneys from the Latter. *Science* **23**, 394-395 (1906).
7. Sterioff, S. ,Rucker-Johnson, N. Frank C. Mann and transplantation at the Mayo Clinic. *Mayo Clin Proc* **62**, 1051-1055 (1987).
8. Schmauss, D. ,Weis, M. Cardiac allograft vasculopathy: recent developments. *Circulation* **117**, 2131-2141 (2008).
9. Hunt, S.A. ,Haddad, F. The changing face of heart transplantation. *J Am Coll Cardiol* **52**, 587-598 (2008).
10. Stehlik, J., Edwards, L.B., Kucheryavaya, A.Y., Benden, C., Christie, J.D., Dipchand, A.I., Dobbels, F., Kirk, R., Rahmel, A.O. ,Hertz, M.I. The registry of the International Society for Heart and Lung Transplantation: 29th official adult heart transplant report--2012. *J Heart Lung Transplant* **31**, 1052-1064 (2012).
11. Zimmer, R.J. ,Lee, M.S. Transplant coronary artery disease. *JACC Cardiovasc Interv* **3**, 367-377 (2010).
12. Chantranuwat, C., Blakey, J.D., Kobashigawa, J.A., Moriguchi, J.D., Laks, H., Vassilakis, M.E. ,Fishbein, M.C. Sudden, unexpected death in cardiac transplant recipients: an autopsy study. *J Heart Lung Transplant* **23**, 683-689 (2004).
13. Park, S.J., Pifarre, R., Sullivan, H., Montoya, A. ,Kim, S.Y. Natural history of allograft coronary arteriopathy: a retrospective study of 54 patients over a 8 1/2-year period. *Cardiovasc Surg* **4**, 37-41 (1996).
14. Perens, G., Li, F., Meier, S., Kaur, R., Alejos, J.C. ,Fishbein, M. Clinico-pathologic findings in end-stage pediatric heart transplant grafts. *Pediatr Transplant* **13**, 887-891 (2009).
15. Johnson, D.E., Gao, S.Z., Schroeder, J.S., DeCampli, W.M. ,Billingham, M.E. The spectrum of coronary artery pathologic findings in human cardiac allografts. *J Heart Transplant* **8**, 349-359 (1989).
16. McManus, B.M., Horley, K.J., Wilson, J.E., Malcom, G.T., Kendall, T.J., Miles, R.R., Winters, G.L., Costanzo, M.R., Miller, L.L. ,Radio, S.J. Prominence of coronary arterial wall lipids in human heart allografts. Implications for pathogenesis of allograft arteriopathy. *Am J Pathol* **147**, 293-308 (1995).

17. Weis, M. ,von Scheidt, W. Coronary artery disease in the transplanted heart. *Annu Rev Med* **51**, 81-100 (2000).
18. Lu, W.H., Palatnik, K., Fishbein, G.A., Lai, C., Levi, D.S., Perens, G., Alejos, J., Kobashigawa, J. ,Fishbein, M.C. Diverse morphologic manifestations of cardiac allograft vasculopathy: a pathologic study of 64 allograft hearts. *J Heart Lung Transplant* **30**, 1044-1050 (2011).
19. Hiemann, N.E., Wellnhofer, E., Knosalla, C., Lehmkuhl, H.B., Stein, J., Hetzer, R. ,Meyer, R. Prognostic impact of microvasculopathy on survival after heart transplantation: evidence from 9713 endomyocardial biopsies. *Circulation* **116**, 1274-1282 (2007).
20. Mairesse, G.H., Marwick, T.H., Melin, J.A., Hanet, C., Jacquet, L., Dion, R. ,Goenen, M. Use of exercise electrocardiography, technetium-99m-MIBI perfusion tomography, and two-dimensional echocardiography for coronary disease surveillance in a low-prevalence population of heart transplant recipients. *J Heart Lung Transplant* **14**, 222-229 (1995).
21. Ciliberto, G.R., Mangiavacchi, M., Banfi, F., Massa, D., Danzi, G., Cataldo, G., Cipriani, M., Piccalo, G., Dabala, A., Gronda, E. ,et al. Coronary artery disease after heart transplantation: non-invasive evaluation with exercise thallium scintigraphy. *Eur Heart J* **14**, 226-229 (1993).
22. Smart, F.W., Ballantyne, C.M., Cocanougher, B., Farmer, J.A., Sekela, M.E., Noon, G.P. ,Young, J.B. Insensitivity of noninvasive tests to detect coronary artery vasculopathy after heart transplant. *Am J Cardiol* **67**, 243-247 (1991).
23. Collings, C.A., Pinto, F.J., Valantine, H.A., Popylisen, S., Puryear, J.V. ,Schnittger, I. Exercise echocardiography in heart transplant recipients: a comparison with angiography and intracoronary ultrasonography. *J Heart Lung Transplant* **13**, 604-613 (1994).
24. Kapadia, S.R., Nissen, S.E. ,Tuzcu, E.M. Impact of intravascular ultrasound in understanding transplant coronary artery disease. *Curr Opin Cardiol* **14**, 140-150 (1999).
25. Kobashigawa, J.A., Tobis, J.M., Starling, R.C., Tuzcu, E.M., Smith, A.L., Valantine, H.A., Yeung, A.C., Mehra, M.R., Anzai, H., Oeser, B.T., Abeywickrama, K.H., Murphy, J. ,Cretin, N. Multicenter intravascular ultrasound validation study among heart transplant recipients: outcomes after five years. *J Am Coll Cardiol* **45**, 1532-1537 (2005).
26. Tuzcu, E.M., Kapadia, S.R., Sachar, R., Ziada, K.M., Crowe, T.D., Feng, J., Magyar, W.A., Hobbs, R.E., Starling, R.C., Young, J.B., McCarthy, P. ,Nissen, S.E. Intravascular ultrasound evidence of angiographically silent progression in coronary atherosclerosis predicts long-term morbidity and mortality after cardiac transplantation. *J Am Coll Cardiol* **45**, 1538-1542 (2005).
27. Nagji, A.S., Hranjec, T., Swenson, B.R., Kern, J.A., Bergin, J.D., Jones, D.R., Kron, I.L., Lau, C.L. ,Ailawadi, G. Donor age is associated with chronic allograft vasculopathy after adult heart transplantation: implications for donor allocation. *Ann Thorac Surg* **90**, 168-175 (2010).

28. Escobar, A., Ventura, H.O., Stapleton, D.D., Mehra, M.R., Ramee, S.R., Collins, T.J., Jain, S.P., Smart, F.W. ,White, C.J. Cardiac allograft vasculopathy assessed by intravascular ultrasonography and nonimmunologic risk factors. *Am J Cardiol* **74**, 1042-1046 (1994).
29. Mehra, M.R., Ventura, H.O., Chambers, R., Collins, T.J., Ramee, S.R., Kates, M.A., Smart, F.W. ,Stapleton, D.D. Predictive model to assess risk for cardiac allograft vasculopathy: an intravascular ultrasound study. *J Am Coll Cardiol* **26**, 1537-1544 (1995).
30. Raichlin, E., Edwards, B.S., Kremers, W.K., Clavell, A.L., Rodeheffer, R.J., Frantz, R.P., Pereira, N.L., Daly, R.C., McGregor, C.G., Lerman, A. ,Kushwaha, S.S. Acute cellular rejection and the subsequent development of allograft vasculopathy after cardiac transplantation. *J Heart Lung Transplant* **28**, 320-327 (2009).
31. Grattan, M.T., Moreno-Cabral, C.E., Starnes, V.A., Oyer, P.E., Stinson, E.B. ,Shumway, N.E. Cytomegalovirus infection is associated with cardiac allograft rejection and atherosclerosis. *Jama* **261**, 3561-3566 (1989).
32. Valantine, H. Cardiac allograft vasculopathy after heart transplantation: risk factors and management. *J Heart Lung Transplant* **23**, S187-193 (2004).
33. Lietz, K. ,Miller, L.W. Current understanding and management of allograft vasculopathy. *Semin Thorac Cardiovasc Surg* **16**, 386-394 (2004).
34. Miller, L.W. Allograft coronary disease: the role of antigen independent mechanisms in pathogenesis. *Ann Transplant* **2**, 61-64 (1997).
35. Kobashigawa, J.A., Katznelson, S., Laks, H., Johnson, J.A., Yeatman, L., Wang, X.M., Chia, D., Terasaki, P.I., Sabad, A., Cogert, G.A. ,et al. Effect of pravastatin on outcomes after cardiac transplantation. *N Engl J Med* **333**, 621-627 (1995).
36. Wenke, K., Meiser, B., Thiery, J., Nagel, D., von Scheidt, W., Steinbeck, G., Seidel, D. ,Reichart, B. Simvastatin reduces graft vessel disease and mortality after heart transplantation: a four-year randomized trial. *Circulation* **96**, 1398-1402 (1997).
37. Rickenbacher, P.R., Kemna, M.S., Pinto, F.J., Hunt, S.A., Alderman, E.L., Schroeder, J.S., Stinson, E.B., Popp, R.L., Chen, I., Reaven, G. ,Valantine, H.A. Coronary artery intimal thickening in the transplanted heart. An in vivo intracoronary untrasound study of immunologic and metabolic risk factors. *Transplantation* **61**, 46-53 (1996).
38. Valantine, H., Rickenbacker, P., Kemna, M., Hunt, S., Chen, Y.D., Reaven, G. ,Stinson, E.B. Metabolic abnormalities characteristic of dysmetabolic syndrome predict the development of transplant coronary artery disease: a prospective study. *Circulation* **103**, 2144-2152 (2001).
39. Grimm, M., Rinaldi, M., Yonan, N.A., Arpesella, G., Arizon Del Prado, J.M., Pulpon, L.A., Villemot, J.P., Frigerio, M., Rodriguez Lambert, J.L., Crespo-Leiro, M.G., Almenar, L., Duveau, D., Ordonez-Fernandez, A., Gandjbakhch, J., Maccherini, M. ,Laufer, G. Superior prevention of acute rejection by tacrolimus vs. cyclosporine in heart transplant recipients--a large European trial. *Am J Transplant* **6**, 1387-1397 (2006).
40. Kobashigawa, J.A., Miller, L.W., Russell, S.D., Ewald, G.A., Zucker, M.J., Goldberg, L.R., Eisen, H.J., Salm, K., Tolzman, D., Gao, J., Fitzsimmons, W. ,First, R. Tacrolimus with

mycophenolate mofetil (MMF) or sirolimus vs. cyclosporine with MMF in cardiac transplant patients: 1-year report. *Am J Transplant* **6**, 1377-1386 (2006).

41. Kobashigawa, J.A., Patel, J., Furukawa, H., Moriguchi, J.D., Yeatman, L., Takemoto, S., Marquez, A., Shaw, J., Oeser, B.T., Subherwal, S., Wu, G.W., Kawano, J. ,Laks, H. Five-year results of a randomized, single-center study of tacrolimus vs microemulsion cyclosporine in heart transplant patients. *J Heart Lung Transplant* **25**, 434-439 (2006).
42. Eisen, H.J., Kobashigawa, J., Keogh, A., Bourge, R., Renlund, D., Mentzer, R., Alderman, E., Valantine, H., Dureau, G., Mancini, D., Mamelok, R., Gordon, R., Wang, W., Mehra, M., Constanzo, M.R., Hummel, M. ,Johnson, J. Three-year results of a randomized, double-blind, controlled trial of mycophenolate mofetil versus azathioprine in cardiac transplant recipients. *J Heart Lung Transplant* **24**, 517-525 (2005).
43. Kobashigawa, J.A., Tobis, J.M., Mentzer, R.M., Valantine, H.A., Bourge, R.C., Mehra, M.R., Smart, F.W., Miller, L.W., Tanaka, K., Li, H., Gjertson, D.W. ,Gordon, R.D. Mycophenolate mofetil reduces intimal thickness by intravascular ultrasound after heart transplant: reanalysis of the multicenter trial. *Am J Transplant* **6**, 993-997 (2006).
44. Kaczmarek, I., Ertl, B., Schmauss, D., Sadoni, S., Knez, A., Daebritz, S., Meiser, B. ,Reichart, B. Preventing cardiac allograft vasculopathy: long-term beneficial effects of mycophenolate mofetil. *J Heart Lung Transplant* **25**, 550-556 (2006).
45. Schaffer, S.A. ,Ross, H.J. Everolimus: efficacy and safety in cardiac transplantation. *Expert Opin Drug Saf* **9**, 843-854 (2010).
46. Eisen, H.J., Tuzcu, E.M., Dorent, R., Kobashigawa, J., Mancini, D., Valantine-von Kaeppler, H.A., Starling, R.C., Sorensen, K., Hummel, M., Lind, J.M., Abeywickrama, K.H. ,Bernhardt, P. Everolimus for the prevention of allograft rejection and vasculopathy in cardiac-transplant recipients. *N Engl J Med* **349**, 847-858 (2003).
47. Arora, S., Ueland, T., Wennerblom, B., Sigurdadottir, V., Eiskjaer, H., Botker, H.E., Ekmehag, B., Jansson, K., Mortensen, S.A., Saunamaki, K., Simonsen, S., Gude, E., Bendz, B., Solbu, D., Aukrust, P. ,Gullestad, L. Effect of everolimus introduction on cardiac allograft vasculopathy--results of a randomized, multicenter trial. *Transplantation* **92**, 235-243 (2011).
48. Keogh, A., Richardson, M., Ruygrok, P., Spratt, P., Galbraith, A., O'Driscoll, G., Macdonald, P., Esmore, D., Muller, D. ,Faddy, S. Sirolimus in de novo heart transplant recipients reduces acute rejection and prevents coronary artery disease at 2 years: a randomized clinical trial. *Circulation* **110**, 2694-2700 (2004).
49. Mancini, D., Pinney, S., Burkhoff, D., LaManca, J., Itescu, S., Burke, E., Edwards, N., Oz, M. ,Marks, A.R. Use of rapamycin slows progression of cardiac transplantation vasculopathy. *Circulation* **108**, 48-53 (2003).
50. Rogers, N.J. ,Lechler, R.I. Allorecognition. *Am J Transplant* **1**, 97-102 (2001).
51. Goulmy, E., Termijtelen, A., Bradley, B.A. ,van Rood, J.J. Alloimmunity to human H-Y. *Lancet* **2**, 1206 (1976).

52. Archbold, J.K., Ely, L.K., Kjer-Nielsen, L., Burrows, S.R., Rossjohn, J., McCluskey, J. ,Macdonald, W.A. T cell allorecognition and MHC restriction--A case of Jekyll and Hyde? *Mol Immunol* **45**, 583-598 (2008).
53. Zinkernagel, R.M. ,Doherty, P.C. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* **248**, 701-702 (1974).
54. Zinkernagel, R.M., Callahan, G.N., Klein, J. ,Dennert, G. Cytotoxic T cells learn specificity for self H-2 during differentiation in the thymus. *Nature* **271**, 251-253 (1978).
55. Matzinger, P., Zamoyska, R. ,Waldmann, H. Self tolerance is H-2-restricted. *Nature* **308**, 738-741 (1984).
56. Game, D.S. ,Lechler, R.I. Pathways of allorecognition: implications for transplantation tolerance. *Transpl Immunol* **10**, 101-108 (2002).
57. Beilke, M.A. Vascular endothelium in immunology and infectious disease. *Rev Infect Dis* **11**, 273-283 (1989).
58. Kreisel, D., Krupnick, A.S., Gelman, A.E., Engels, F.H., Popma, S.H., Krasinskas, A.M., Balsara, K.R., Szeto, W.Y., Turka, L.A. ,Rosengard, B.R. Non-hematopoietic allograft cells directly activate CD8+ T cells and trigger acute rejection: an alternative mechanism of allorecognition. *Nat Med* **8**, 233-239 (2002).
59. Choi, J., Enis, D.R., Koh, K.P., Shiao, S.L. ,Pober, J.S. T lymphocyte-endothelial cell interactions. *Annu Rev Immunol* **22**, 683-709 (2004).
60. Kapessidou, Y., Habran, C., Buonocore, S., Flamand, V., Barvais, L., Goldman, M. ,Braun, M.Y. The replacement of graft endothelium by recipient-type cells conditions allograft rejection mediated by indirect pathway CD4+ T cells. *Transplantation* **82**, 582-591 (2006).
61. Weis, M., Schlichting, C.L., Engleman, E.G. ,Cooke, J.P. Endothelial determinants of dendritic cell adhesion and migration: new implications for vascular diseases. *Arterioscler Thromb Vasc Biol* **22**, 1817-1823 (2002).
62. Behrendt, D. ,Ganz, P. Endothelial function. From vascular biology to clinical applications. *Am J Cardiol* **90**, 40L-48L (2002).
63. Vassalli, G., Gallino, A., Weis, M., von Scheidt, W., Kappenberger, L., von Segesser, L.K. ,Goy, J.J. Alloimmunity and nonimmunologic risk factors in cardiac allograft vasculopathy. *Eur Heart J* **24**, 1180-1188 (2003).
64. van Loosdregt, J., van Oosterhout, M.F., Bruggink, A.H., van Wichen, D.F., van Kuik, J., de Koning, E., Baan, C.C., de Jonge, N., Gmelig-Meyling, F.H. ,de Weger, R.A. The chemokine and chemokine receptor profile of infiltrating cells in the wall of arteries with cardiac allograft vasculopathy is indicative of a memory T-helper 1 response. *Circulation* **114**, 1599-1607 (2006).
65. Vasa, M., Fichtlscherer, S., Adler, K., Aicher, A., Martin, H., Zeiher, A.M. ,Dimmeler, S. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation* **103**, 2885-2890 (2001).

66. Hill, J.M., Zalos, G., Halcox, J.P., Schenke, W.H., Waclawiw, M.A., Quyyumi, A.A. ,Finkel, T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* **348**, 593-600 (2003).

67. Hirschi, K.K., Ingram, D.A. ,Yoder, M.C. Assessing identity, phenotype, and fate of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* **28**, 1584-1595 (2008).

68. Urbich, C., Aicher, A., Heeschen, C., Dernbach, E., Hofmann, W.K., Zeiher, A.M. ,Dimmeler, S. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. *J Mol Cell Cardiol* **39**, 733-742 (2005).

69. Feng, Y., Jacobs, F., Van Craeyveld, E., Brunaud, C., Snoeys, J., Tjwa, M., Van Linthout, S. ,De Geest, B. Human ApoA-I transfer attenuates transplant arteriosclerosis via enhanced incorporation of bone marrow-derived endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* **28**, 278-283 (2008).

70. Feng, Y., Gordts, S.C., Chen, F., Hu, Y., Van Craeyveld, E., Jacobs, F., Carlier, V., Zhang, Z., Xu, Q., Ni, Y. ,De Geest, B. Topical HDL administration reduces vein graft atherosclerosis in apo E deficient mice. *Atherosclerosis* **214**, 271-278 (2011).

71. Ingram, D.A., Mead, L.E., Tanaka, H., Meade, V., Fenoglio, A., Mortell, K., Pollok, K., Ferkowicz, M.J., Gilley, D. ,Yoder, M.C. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood* **104**, 2752-2760 (2004).

72. Wara, A.K., Croce, K., Foo, S., Sun, X., Icli, B., Tesmenitsky, Y., Esen, F., Rosenzweig, A. ,Feinberg, M.W. Bone marrow-derived CMPs and GMPs represent highly functional pro-angiogenic cells: implications for ischemic cardiovascular disease. *Blood* (2011).

73. Erdbruegger, U., Haubitz, M. ,Woywodt, A. Circulating endothelial cells: a novel marker of endothelial damage. *Clin Chim Acta* **373**, 17-26 (2006).

74. Dignat-George, F. ,Sampol, J. Circulating endothelial cells in vascular disorders: new insights into an old concept. *Eur J Haematol* **65**, 215-220 (2000).

75. Woywodt, A., Bahlmann, F.H., De Groot, K., Haller, H. ,Haubitz, M. Circulating endothelial cells: life, death, detachment and repair of the endothelial cell layer. *Nephrol Dial Transplant* **17**, 1728-1730 (2002).

76. van Engeland, M., Nieland, L.J., Ramaekers, F.C., Schutte, B. ,Reutelingsperger, C.P. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* **31**, 1-9 (1998).

77. Faure, V., Dou, L., Sabatier, F., Cerini, C., Sampol, J., Berland, Y., Brunet, P. ,Dignat-George, F. Elevation of circulating endothelial microparticles in patients with chronic renal failure. *J Thromb Haemost* **4**, 566-573 (2006).

78. Simper, D., Wang, S., Deb, A., Holmes, D., McGregor, C., Frantz, R., Kushwaha, S.S. ,Caplice, N.M. Endothelial progenitor cells are decreased in blood of cardiac allograft patients with vasculopathy and endothelial cells of noncardiac origin are enriched in transplant atherosclerosis. *Circulation* **108**, 143-149 (2003).

79. Thomas, H.E., Parry, G., Dark, J.H., Arthur, H.M. ,Keavney, B.D. Circulating endothelial progenitor cell numbers are not associated with donor organ age or allograft vasculopathy in cardiac transplant recipients. *Atherosclerosis* **202**, 612-616 (2009).

80. Schober, A., Hristov, M., Kofler, S., Forbrig, R., Lohr, B., Heussen, N., Zhe, Z., Akhtar, S., Schumann, U., Krotz, F., Leibig, M., Konig, A., Kaczmarek, I., Reichart, B., Klauss, V., Weber, C. ,Sohn, H.Y. CD34+CD140b+ cells and circulating CXCL12 correlate with the angiographically assessed severity of cardiac allograft vasculopathy. *Eur Heart J* **32**, 476-484 (2011).

81. O'Connell, J.B., Renlund, D.G., Bristow, M.R. ,Hammond, E.H. Detection of allograft endothelial cells of recipient origin following ABO-compatible, nonidentical cardiac transplantation. *Transplantation* **51**, 438-442 (1991).

82. Koestner, S.C., Kappeler, A., Schaffner, T., Carrel, T.P., Nydegger, U.E. ,Mohacsi, P. Histo-blood group type change of the graft from B to O after ABO mismatched heart transplantation. *Lancet* **363**, 1523-1525 (2004).

83. West, L.J., Pollock-Barziv, S.M., Lee, K.J., Dipchand, A.I., Coles, J.G. ,Ruiz, P. Graft accommodation in infant recipients of ABO-incompatible heart transplants: donor ABH antigen expression in graft biopsies. *J Heart Lung Transplant* **20**, 222 (2001).

84. D'Agostino, R.B., Sr., Grundy, S., Sullivan, L.M. ,Wilson, P. Validation of the Framingham coronary heart disease prediction scores: results of a multiple ethnic groups investigation. *Jama* **286**, 180-187 (2001).

85. Conroy, R.M., Pyorala, K., Fitzgerald, A.P., Sans, S., Menotti, A., De Backer, G., De Bacquer, D., Ducimetiere, P., Jousilahti, P., Keil, U., Njolstad, I., Oganov, R.G., Thomsen, T., Tunstall-Pedoe, H., Tverdal, A., Wedel, H., Whincup, P., Wilhelmsen, L. ,Graham, I.M. Estimation of ten-year risk of fatal cardiovascular disease in Europe: the SCORE project. *Eur Heart J* **24**, 987-1003 (2003).

86. Moons, K.G. Criteria for scientific evaluation of novel markers: a perspective. *Clin Chem* **56**, 537-541 (2010).

Chapter 2

Objectives

Cardiac allograft vasculopathy (CAV) is a limiting factor for the long-term survival of heart transplant recipients[1,2]. CAV is characterized by the development of diffuse concentric fibromuscular intimal hyperplasia lesions in epicardial and smaller intramyocardial arteries along with focal, eccentric atherosclerotic plaques in the larger epicardial arteries[3,4]. The development of these lesions may lead to the progressive narrowing of the lumen[5]. According to the response to injury hypothesis of CAV, these lesions are the result of cumulative endothelial injury induced by alloimmune responses as well as non-immunological risk factors such as ischemia-reperfusion injury, viral infections, and metabolic disorders[3,6].
Early diagnosis of CAV is essential to implement appropriate prevention and treatment measures. Clinical prediction models of CAV are currently not available and may be useful for non-invasive diagnostic and prognostic purposes. The general aim of this doctoral thesis is to develop diagnostic prediction models for prevalent CAV. The central hypothesis of my PhD studies is that biomarkers of endothelial homeostasis discriminate between CAV negative and CAV positive heart transplant recipients. The validity of this hypothesis will be specifically demonstrated in chapter 3.1 and chapter 3.4.

The precise aims of the four chapters comprised in this doctoral thesis can be summarized as follows:

1. The hypothesis evaluated in chapter 3.1 was that biomarkers of endothelial homeostasis would constitute a solid foundation for the development of clinical prediction models of CAV. Therefore, the objective of the first study was to evaluate whether biomarkers related to endothelial repair (endothelial progenitor cell (EPC) number, EPC function, hematopoietic progenitor cell (HPC) number) and to endothelial injury ((apoptotic) circulating endothelial cells (CECs), (apoptotic) circulating endothelial microparticles (CEMPs)) discriminate between CAV negative and CAV positive heart transplant recipients. In this cross-sectional study, 22 CAV negative patients and 30 positive patients between 5 and 15 years after heart transplantation were included.

2. The aim of the second cross-sectional study was to compare CECs, CEMPs, and platelet microparticles (PMPs) between heart transplant recipients and patients with stable native coronary artery disease (CAD). After all, according to the response to injury hypothesis of Russell Ross[7,8], endothelial dysfunction/injury induced by various insults is the trigger for atherosclerosis development. To perform this study, 80 patients undergoing coronary angiography for stable native CAD were recruited. Since CAV and native CAD are two entirely distinct categories, the main objective of this study was to test the hypothesis that biomarkers of endothelial injury (endothelial activation or endothelial damage) are distinct in patients with native CAD and heart transplant recipients with CAV.

3. The relation between high density lipoprotein (HDL) cholesterol levels and CAV is controversial in the literature. In this cross-sectional study, the hypothesis that HDL is dysfunctional in heart

transplant recipients was investigated. Heart transplant recipients were compared with a healthy control reference group. Moreover, the objective of this study was to evaluate whether HDL function assays may discriminate between CAV negative and CAV positive heart transplant recipients. To assess cholesterol efflux capacity of HDL, we used a validated assay that was designed to integrate the efflux pathways thought to be operative *in vivo*[9]. Cholesterol efflux capacity analyzed by this assay was a stronger predictor of prevalent atherosclerotic burden than HDL cholesterol or apolipoprotein (apo) A-I in the study by Khera *et al*.[9]. Since cumulative endothelial injury induced by both alloimmune responses and non-alloimmune insults is thought to be central in the pathogenesis of CAV[2,10], we also assessed the vasculoprotective function of HDL by use of an EPC migration assay[11,12].

4. The aim of chapter 3.4 was to analyze the potential of endothelium-enriched microRNAs (miRNAs) as putative biomarkers for the prediction of CAV. MiRNAs are small, non-coding, single-stranded RNA sequences that regulate gene expression at the post-transcriptional level. Because miRNAs circulate in remarkably stable forms in blood[13,14], they have a significant potential as biomarkers. Several reports indicate that miRNAs may play a role in endothelial homeostasis[15,16]. In this study, a candidate-based approach using circulating levels of endothelium-enriched miRNAs (miR-21-5p, miR-92a-3p, miR-92a-1-5p, miR-126-3p, miR-126-5p) to predict CAV was investigated.

2.1 REFERENCES

1. Stehlik, J., Edwards, L.B., Kucheryavaya, A.Y., Benden, C., Christie, J.D., Dobbels, F., Kirk, R., Rahmel, A.O. ,Hertz, M.I. The Registry of the International Society for Heart and Lung Transplantation: Twenty-eighth Adult Heart Transplant Report--2011. *J Heart Lung Transplant* **30**, 1078-1094 (2011).
2. Schmauss, D. ,Weis, M. Cardiac allograft vasculopathy: recent developments. *Circulation* **117**, 2131-2141 (2008).
3. Vassalli, G., Gallino, A., Weis, M., von Scheidt, W., Kappenberger, L., von Segesser, L.K. ,Goy, J.J. Alloimmunity and nonimmunologic risk factors in cardiac allograft vasculopathy. *Eur Heart J* **24**, 1180-1188 (2003).
4. Rahmani, M., Cruz, R.P., Granville, D.J. ,McManus, B.M. Allograft vasculopathy versus atherosclerosis. *Circ Res* **99**, 801-815 (2006).
5. Kapadia, S.R., Nissen, S.E. ,Tuzcu, E.M. Impact of intravascular ultrasound in understanding transplant coronary artery disease. *Curr Opin Cardiol* **14**, 140-150 (1999).
6. Ross, R. The pathogenesis of atherosclerosis--an update. *N Engl J Med* **314**, 488-500 (1986).
7. Ross, R., Glomset, J. ,Harker, L. Response to injury and atherogenesis. *Am J Pathol* **86**, 675-684 (1977).
8. Ross, R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* **362**, 801-809. (1993).
9. Khera, A.V., Cuchel, M., de la Llera-Moya, M., Rodrigues, A., Burke, M.F., Jafri, K., French, B.C., Phillips, J.A., Mucksavage, M.L., Wilensky, R.L., Mohler, E.R., Rothblat, G.H. ,Rader, D.J. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *N Engl J Med* **364**, 127-135 (2011).
10. Vassalli, G., Gallino, A., Weis, M., von Scheidt, W., Kappenberger, L., von Segesser, L.K. ,Goy, J.J. Alloimmunity and nonimmunologic risk factors in cardiac allograft vasculopathy. *Eur Heart J* **24**, 1180-1188 (2003).
11. Smadja, D.M., Bieche, I., Uzan, G., Bompais, H., Muller, L., Boisson-Vidal, C., Vidaud, M., Aiach, M. ,Gaussem, P. PAR-1 activation on human late endothelial progenitor cells enhances angiogenesis in vitro with upregulation of the SDF-1/CXCR4 system. *Arterioscler Thromb Vasc Biol* **25**, 2321-2327 (2005).
12. Feng, Y., Jacobs, F., Van Craeyveld, E., Brunaud, C., Snoeys, J., Tjwa, M., Van Linthout, S. ,De Geest, B. Human ApoA-I transfer attenuates transplant arteriosclerosis via enhanced incorporation of bone marrow-derived endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* **28**, 278-283 (2008).
13. Suarez, Y., Fernandez-Hernando, C., Yu, J., Gerber, S.A., Harrison, K.D., Pober, J.S., Iruela-Arispe, M.L., Merkenschlager, M. ,Sessa, W.C. Dicer-dependent endothelial microRNAs are necessary for postnatal angiogenesis. *Proc Natl Acad Sci U S A* **105**, 14082-14087 (2008).

14. Kuehbacher, A., Urbich, C., Zeiher, A.M. ,Dimmeler, S. Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. *Circ Res* **101**, 59-68 (2007).
15. Scott, E., Loya, K., Mountford, J., Milligan, G. ,Baker, A.H. MicroRNA regulation of endothelial homeostasis and commitment-implications for vascular regeneration strategies using stem cell therapies. *Free Radic Biol Med* **64**, 52-60 (2013).
16. Yamakuchi, M. MicroRNAs in Vascular Biology. *Int J Vasc Med* **2012**, 794898 (2012).

Chapter 3

Experimental studies

3.1 CIRCULATING APOPTOTIC ENDOTHELIAL CELLS AND APOPTOTIC ENDOTHELIAL MICROPARTICLES INDEPENDENTLY PREDICT THE PRESENCE OF CARDIAC ALLOGRAFT VASCULOPATHY*

3.1.1 ABSTRACT

Objectives: Maintenance of endothelial homeostasis may prevent the development of cardiac allograft vasculopathy (CAV). This study investigated whether biomarkers related to endothelial injury and endothelial repair discriminate between CAV-negative and CAV-positive heart transplant recipients.

Background: CAV is the most important determinant of cardiac allograft survival and a major cause of death after heart transplantation.

Methods: Fifty-two patients undergoing coronary angiography between 5 and 15 years after heart transplantation were recruited in this study. Flow cytometry was applied to quantify endothelial progenitor cells (EPCs), circulating endothelial cells (CECs), and endothelial microparticles. Cell culture was used for quantification of circulating EPC number and hematopoietic progenitor cell (HPC) number and for analysis of EPC function.

Results: The EPC number and function did not differ between CAV-negative and CAV-positive patients. In univariable models, age, creatinine, steroid dose, granulocyte colony-forming units, apoptotic CECs, and apoptotic endothelial microparticles discriminated between CAV-positive and CAV-negative patients. The logistic regression model containing apoptotic CECs and apoptotic endothelial microparticles as independent predictors provided high discrimination between CAV-positive and CAV-negative patients (C-statistic 0.812; 95% confidence interval: 0.692 to 0.932). In a logistic regression model with age and creatinine as covariates, apoptotic CECs (p=0.0112) and apoptotic endothelial microparticles (p=0.0141) were independent predictors (C-statistic 0.855; 95% confidence interval: 0.756 to 0.953). These two biomarkers remained independent predictors when steroid dose was introduced in the model.

Conclusions: The high discriminative ability of apoptotic CECs and apoptotic endothelial microparticles is a solid foundation for the development of clinical prediction models of CAV.

---

* This chapter has been published as: **Singh, N**., Van Craeyveld, E., Tjwa, M., Ciarka, A., Emmerechts, J., Droogne, W., Gordts, S., Carlier, V., Jacobs, F., Fieuws, S., Vanhaecke, J., Van Cleemput, J., De Geest, B. Circulating apoptotic endothelial cells and apoptotic endothelial microparticles independently predict the presence of cardiac allograft vasculopathy. *Journal of the American College of Cardiology* 2012; 60 (4), 324-331

3.1.2 INTRODUCTION

Cardiac allograft vasculopathy (CAV) in heart transplant recipients is characterised by the coexistence of diffuse fibromuscular intimal hyperplasia and focal atherosclerosis[1,2]. It is largely an immunological phenomenon that is modified by non-immunological factors. Diffuse concentric lesions of fibromuscular intimal hyperplasia develop in the epicardial and the smaller intramyocardial arteries whereas focal, eccentric atherosclerotic plaques are observed in the larger epicardial arteries[1,2]. CAV may lead to late graft failure and is, in addition to malignancy, the most important cause of death in heart transplant recipients after the first year[1,3,4].

According to the response to injury concept of CAV, vascular lesions are considered to be the result of cumulative endothelial injury by both alloimmune responses and non-alloimmune insults[1,5]. T-cell alloimmunity, antibody-mediated immune attack, and non-immune factors induce endothelial activation or endothelial cell death. This may initiate a cascade of events including platelet activation and inflammation with infiltration of predominantly macrophages and T cells in the vessel wall followed by smooth muscle cell activation, migration, and proliferation.

To maintain endothelial homeostasis, endothelial cell death should be balanced by endothelial repair mechanisms. After endothelial cell detachment induced by prolonged activation of endothelial cells or immunological injury, endothelial cells can be detected in the peripheral blood as viable circulating endothelial cells (CECs) or as apoptotic CECs[6,7]. In addition to CECs, endothelial microparticles constitute another biomarker of endothelial injury. Endothelial microparticles arise from exocytic budding after endothelial cell activation or apoptosis[8-10]. The process of endothelial injury is counteracted by endothelial repair mechanisms. Increased endothelial progenitor cell (EPC) number and function may enhance endothelial repair in allografts, directly via increased EPC incorporation (‘building block” role) or indirectly via production of growth factors (paracrine role). EPCs that promote re-endothelialization in a paracrine way have been named pro-angiogenic progenitor cells[11] and are in fact hematopoietic lineage cells. Cells of the hematopoietic lineage may be mobilized from the bone marrow and are entrapped in peripheral tissues, where they release angiogenic signals[12]. A comprehensive analysis of endothelial repair mechanisms should therefore not be restricted to classical EPC quantifications but also entail enumeration of hematopoietic progenitor cells (HPCs).

Clinical prediction models of CAV are currently not available and may be useful for noninvasive diagnostic and prognostic purposes. Our hypothesis was that biomarkers of endothelial homeostasis would constitute a solid foundation for the development of such clinical prediction models. Therefore, the objective of the current study was to evaluate whether biomarkers related to endothelial repair (EPC number, EPC function, HPC number) and to endothelial injury ((apoptotic) CECs, (apoptotic) endothelial microparticles)) discriminate between CAV-negative and CAV-positive heart transplant recipients.

3.1.3 METHODS

3.1.3.1 Patient population and CAV grading

Fifty-two patients undergoing coronary angiography in the framework of their routine follow-up between 5 and 15 years after heart transplantation were recruited in this cross-sectional study. Heart transplant recipients with prior congenital heart disease and patients who underwent retransplantation were excluded. The study was approved by the Ethics Committee of the University Hospital Gasthuisberg and written informed consent was obtained from all participating subjects. To establish reference values of selected biomarkers, 25 healthy control subjects age 43.2 $\pm$ 2.0 years were selected.

Coronary angiograms were analyzed by three transplant cardiologists (A.C., W.D., and J.V.C.). CAV was graded according to the International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for CAV-2010[13]. Patients with CAV_1, CAV_2, and CAV_3 were pooled and constitute the CAV-positive group. Patients with CAV_0, defined as no detectable angiographic lesion, constitute the CAV-negative group.

Endomyocardial biopsies were scored for acute rejection according to Billingham *et al*.[14] and mean biopsy score of all biopsies in the first year was calculated according to Mehra *et al*.[15].

3.1.3.2 Quantification of EPC number and EPC function based on cell culture assays

Peripheral blood was anticoagulated with sodium heparin and mononuclear cells were isolated from 18 ml of blood by density gradient centrifugation with Ficoll-Paque™ PLUS (Stem Cell Technologies, Grenoble, France), according to the manufacturer's protocol. Mononuclear cell count was determined using a Nucleocounter (Chemometec, Allerød, Denmark).

Cultivation of early EPCs was performed as described by Vasa *et al*.[16]. Briefly, mononuclear cells were plated onto fibronectin-coated 24-well culture dishes (BD Biosciences, San Jose, CA, USA) in endothelial basal medium (EBM; Cambrex, East Rutherford, NJ, USA) supplemented with endothelial growth medium SingleQuots (Cambrex) and 20% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at a density of 4 x 10^6 cells/well. After 4 days of incubation, EPCs were quantified as the number of 1,1'-dioctadecyl-3,3,3',3'- tetramethylindocarbocyanine-labeled acetylated LDL (DiI-acLDL) FITC-labeled *Ulex europaeus* agglutinin-I (UEA-I) lectin double positive cells, per microscopy field. Experiments were performed in duplicate.

Migration of cultured early EPCs was studied as previously described[16,17]. Stromal derived factor-1α (SDF-1α) (100 ng/ml; R&D Systems, Inc., Minneapolis, MN, USA) was added in the lower chamber.

3.1.3.3 HPC culture assay

Cultivation of HPCs was performed using semisolid methylcellulose-based medium (Methocult®

H4434 Classic; Stem Cell Technologies), according to the manufacturer's protocol. Briefly, peripheral blood mononuclear cells (1×10^5 cells/dish and 2×10^5 cells/dish) were seeded in 35-mm culture dishes (Stem Cell Technologies) in 2 ml of Methocult medium (Stem Cell Technologies). After 14 days of incubation, erythroid burst-forming units, erythroid colony-forming units, granulocyte colony-forming units, macrophage colony-forming units, granulocyte, macrophage colony-forming units; and granulocyte, erythrocyte, macrophage, megakaryocyte colony-forming units were enumerated using an inverted microscope.

3.1.3.4 Quantification of the number of circulating EPCs, endothelial cells, and endothelial microparticles by flow cytometry

EPC concentration was also measured by fluorescence activated cell sorting analysis of the circulating number of CD34 vascular endothelial growth factor receptor (VEGFR)-2 double positive cells, as described previously[18,19].

CECs were identified as $CD45^-$ $CD31^{bright}$ $VEGFR\text{-}2^+$ mononuclear cells[20]. Annexin V staining distinguishes between viable and apoptotic CECs[21]. Samples were acquired on a high flow rate (120 µl/min) for 3 min using a BD FACSCantoII flow cytometer and BD FACSDIVA software version 1.2.6 (BD Biosciences), with a minimum detection number of 100,000 events within the mononuclear cell gate.

Blood that was used for microparticle quantification by flow cytometry was anticoagulated with sodium citrate. To exclude microparticles derived from nonendothelial cells, mainly platelets, endothelial microparticles were defined as CD144 $(VE\text{-}Cadherin)^+$ $CD42a^-$ microparticles[22]. Annexin V binding was used to discriminate between apoptotic and nonapoptotic microparticles. Apoptotic endothelial microparticles were defined as Annexin V^+ $CD144^+$ $CD42a^-$ microparticles[22].

3.1.3.5 Statistical analysis

Clinical and biochemical parameters and biomarkers (EPC concentration, EPC function, HPC number, CEC concentration, and number of endothelial microparticles) were compared between CAV negative and CAV positive patients using Instat 3 (GraphPad Software, Inc., San Diego, CA, USA). Continuous variables were summarized by means, standard error of the mean, and sample size, and were compared by an unpaired t test. Because the distribution of the concentration of CECs is heavily right-skewed, a transformation (natural logarithm) was applied and geometric means were compared. The Fisher exact test was used to compare categorical data between patients with CAV and without CAV. Logistic regression analysis was performed by SAS software, version 9.2 (SAS Institute Inc., Cary, NC, USA). The discriminative ability is quantified using the concordance statistic (c-statistic), which is equal to the area under the receiver-operating characteristic curve. A natural logarithm transformation of CEC data was also applied for logistic regression analysis. A p value <0.05 was considered statistically significant.

3.1.4 RESULTS

3.1.4.1 Clinical characteristics of heart transplant recipients with and without CAV

The clinical characteristics of heart transplant recipients are shown in Table 3.1.1. Patients with CAV were significantly older at the time of inclusion in the study. There was a trend for a greater donor age in patients with CAV, whereas the difference in time after transplantation did not reach statistical significance. Donor age correlated moderately with the age of the recipient at the time of transplantation ($r=0.368$; $p<0.01$). The age of the transplanted heart (sum of donor age and time after heart transplantation) was significantly greater in CAV-positive patients (Table 3.1.1). The proportion of patients with ischemic heart disease did not differ between both groups. Acute rejection episodes (grade $\geq$3A) were not significantly more frequent in the CAV group than in the non-CAV group (Table 3.1.1). The average rejection score of biopsies in the first year also was not significantly different between patients without CAV (0.484 ± 0.057) and patients with CAV (0.546 ± 0.057). Creatinine levels were significantly greater in the CAV group than in the non-CAV group (Table 3.1.1). Low-density lipoprotein cholesterol in the CAV group was 16% ($p<0.05$) less than in the non-CAV group (Table 3.1.1), which reflects a policy to switch to more potent statins once a diagnosis of angiographic CAV is made. The percentage of patients receiving steroids was 3.5-fold ($p<0.01$) greater in patients with CAV compared to patients without CAV.

3.1.4.2 EPC number and function do not significantly differ between CAV-negative and CAV-positive heart transplant recipients

EPCs were quantified either as the number of DiI-acLDL and FITC-labeled UEA-1 lectin double positive cells after 4 days of *ex vivo* culture or as the number of circulating $CD34^+$ $VEGFR\text{-}2^+$ cells. Gating strategy and flow cytometry analysis of EPCs are illustrated in Figure 3.1.1. The number of FITC labeled UEA-1 lectin double-positive cells was 49% ($p<0.0001$) less in transplant recipients than in healthy controls (Figure 3.1.2A) but did not differ between patients without CAV and with CAV (Table 3.1.2). The number of circulating $CD34^+$ $VEGFR\text{-}2^+$ cells was similar in healthy controls and transplant patients (Figure 3.1.2B) and between CAV-negative and CAV-positive transplant recipients (Table 3.1.2).

EPC migration induced by stromal-derived factor-1α (100 ng/ml) was 31% ($p<0.05$) less in patients than in healthy controls (Figure 3.1.2C). No difference in EPC migration was observed between CAV-negative and CAV-positive patients (Table 3.1.2), indicating similar EPC function.

Table 3.1.1. Patient characteristics, clinical laboratory parameters, and immunosuppressive and hypolipidemic therapy in CAV-negative and CAV-positive heart transplant recipients.

	Patients without CAV (n=22)	Patients with CAV (n=30)	P value
Age at inclusion in the study (years)	56.3 ± 3.2	64.9 ± 2.1	0.0212
Sex			1.00
male	81.8%	80.0%	
female	18.2%	20.0%	
Donor age (years)	33.8 ± 2.9	40.8 ± 2.4	0.0699
Time after heart transplantation (years)	9.35 ± 0.58	10.9 ± 0.8	0.118
Age of the transplanted heart (years)	43.2 ± 3.0	51.7 ± 2.3	0.0298
Sex mismatch graft (%)	13.6%	20.0%	0.716
Acute rejection episodes (Grade 3A or > 3A)	13.6%	10.0%	0.689
Ischemic heart disease (%)	36.4%	50.0%	0.402
Current smoker (%)	13.6%	0%	0.0697
Hypertension (%)	90.9%	100%	0.174
Diabetes (%)	18.2%	36.7%	0.217
Body mass index (kg/m^2)	26.6 ± 0.8	25.5 ± 0.5	0.264
Platelet count (10^9/L)	232 ± 16	217 ± 12	0.421
Leukocyte count (10^9/L)	6.81 ± 0.33	6.87 ± 0.24	0.764
Monocyte count (10^9/L)	0.334 ± 0.048	0.313 ± 0.039	0.704
Lymphocyte count (10^9/L)	1.24 ± 0.13	1.28 ± 0.12	0.793
Neutrophil count (10^9/L)	3.12 ± 0.32	3.78 ± 0.36	0.170
Creatinine (mg/dl)	1.32 ± 0.10	1.60 ± 0.08	0.0134
Cholesterol (mg/dl)	168 ± 8	156 ± 6	0.211
Triglycerides (mg/dl)	108 ± 7	125 ± 12	0.244
HDL cholesterol (mg/dl)	55.0 ± 2.9	54.2 ± 3.4	0.487
LDL cholesterol (mg/dl)	91.6 ± 5.7	77.1 ± 4.0	0.0436
Statins (%)	95.5%	100%	0.423
Cyclosporine (%)	31.8%	23.3%	0.540
Tacrolimus (%)	63.6%	70.0%	0.766
Everolimus (%)	4.55%	20.0%	0.216
Azathioprine (%)	9.10%	13.3%	1.00
Mycophenolate mofetil (%)	86.4%	60.0%	0.0622
Steroid (%)	18.2%	63.3%	0.0018
Cyclosporine+ Azathioprine (%)	9.09%	0%	0.174
Cyclosporine+MMF (%)	22.7%	13.3%	0.468
Tacrolimus+MMF (%)	59.1%	40.0%	0.160
Everolimus+MMF (%)	4.55%	16.7%	0.225
Tacrolimus+Everolimus (%)	0%	10.0%	0.253

Data of continuous variables represent means ± SEM.

3.1.4.3 Granulocyte colony-forming units are significantly less in the peripheral blood of patients with CAV than in patients without CAV

Figure 3.1.3 compares HPC number between healthy controls and transplant recipients. Erythroid burst-forming units, granulocyte, macrophage colony-forming units, macrophage colony-forming units, and granulocyte colony-forming units were significantly less in transplant recipients than in healthy controls, whereas no significant differences were observed for erythroid colony-forming units and granulocyte, erythrocyte, macrophage, megakaryocyte colony-forming units (Figure 3.1.3). The number of granulocyte colony-forming units was reduced by 59% ($p<0.05$) in patients with CAV compared to patients without CAV but no significant differences were observed in other types of colonies (Table 3.1.2).

Table 3.1.2. Comparison of EPC number, EPC function, and HPC number between CAV-negative and CAV-positive patients.

	Patients without CAV (n=22)	Patients with CAV (n=30)	P value
Dil-acLDL / lectin$^+$ cells (number/mm^2)	13.6 ± 1.9	14.6 ± 1.8	0.685
CD34$^+$ VEGFR2$^+$ cells (number/µl blood)	2.06 ± 0.46	1.95 ± 0.52	0.875
Migrated cells (number/mm^2)	38.4 ± 3.7	35.1 ± 3.1	0.495
CFU-E (number per 2 x 10^5 MNC)	5.20 ± 0.81	5.08 ± 0.81	0.910
BFU-E (number per 2 x 10^5 MNC)	17.1 ± 2.1	21.6 ± 2.6	0.180
CFU-GEMM (number per 2 x 10^5 MNC)	1.18 ± 0.29	2.08 ± 0.58	0.179
CFU-GM (number per 2 x 10^5 MNC)	3.91 ± 0.61	3.53 ± 0.49	0.634
CFU-M (number per 2 x 10^5 MNC)	0.750 ± 0.157	0.975 ± 0.342	0.554
CFU-G (number per 2 x 10^5 MNC)	0.523 ± 0.125	0.217 ± 0.089	0.0147

CFU-E: erythroid colony-forming units; BFU-E: erythroid burst-forming units; CFU-GEMM: granulocyte, erythrocyte, macrophage, megakaryocyte colony-forming units; CFU-GM: granulocyte, macrophage colony-forming units; CFU-M: macrophage colony-forming units; CFU-G: granulocyte colony-forming units; MNC: mononuclear cells. All data represent means ± SEM.

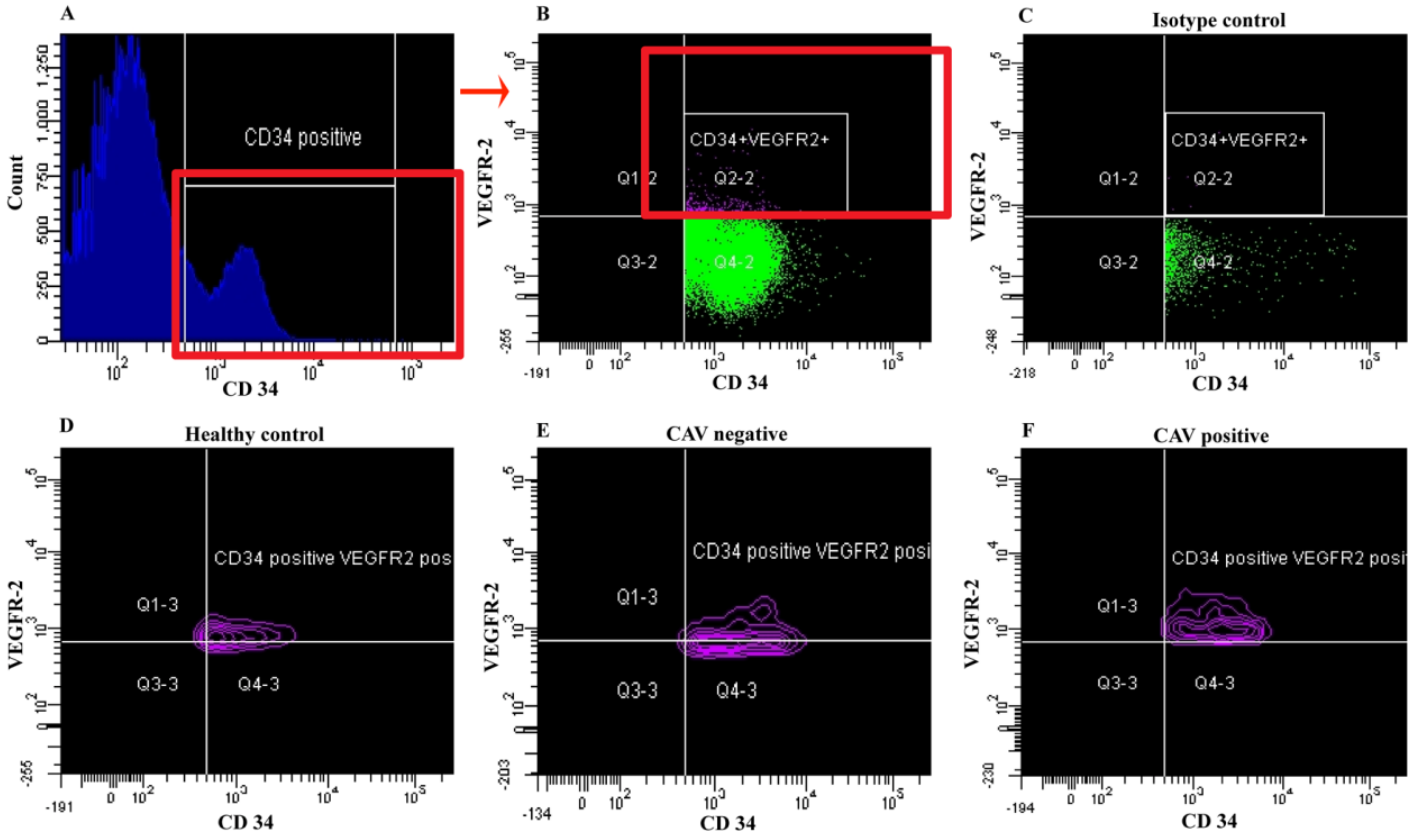

Figure 3.1.1. Flow cytometry analysis of EPCs. Gating strategy for EPCs, identified as $CD34^+$ $VEGFR\text{-}2^+$ mononuclear cells in whole blood samples (A, B). The first gate is set on $CD34^+$ cells out of the mononuclear cellular events (A), which is then displayed on a scatter plot to quantify $CD34^+$ $VEGFR\text{-}2^+$ cells (B). The isotype control for VEGFR-2 is shown in panel C. Representative contour plots of EPCs in healthy controls, CAV negative transplant recipients, and CAV positive transplant recipients are shown in panels D, E, and F, respectively.

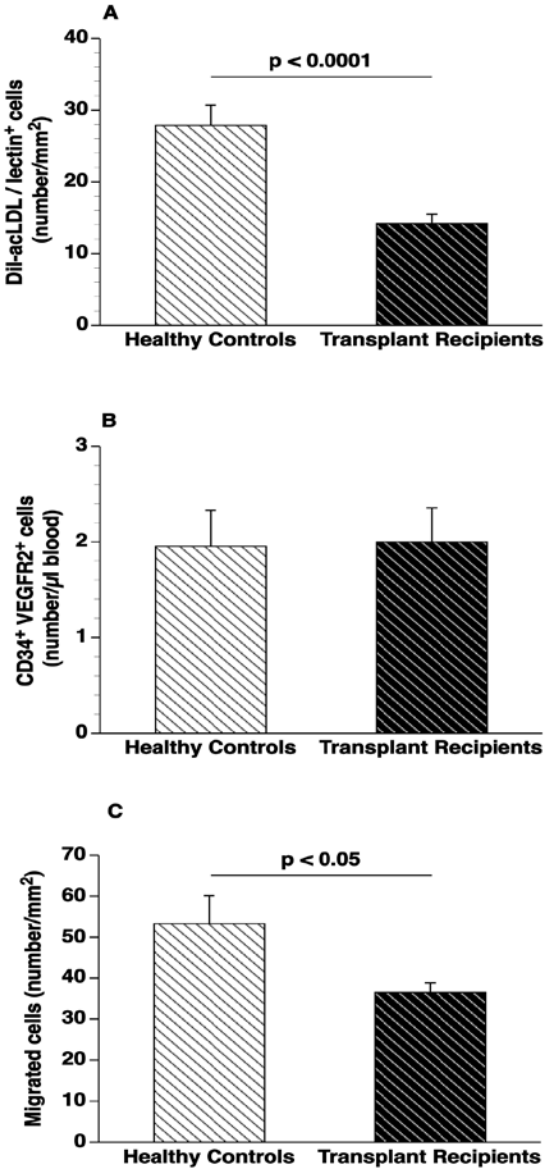

Figure 3.1.2. Quantification of EPC number and EPC function in healthy controls and transplant recipients. EPC number was quantified as the number of Dil-acLDL and FITC labeled UEA-1 lectin double positive cells (A) or as the concentration of circulating $CD34^+$ $VEGFR2^+$ cells (B). EPC function was evaluated by quantification of EPC migration in modified Boyden chambers (C). Comparisons were made between healthy controls (n=25) and transplant recipients (n=52). All data represent means ± SEM.

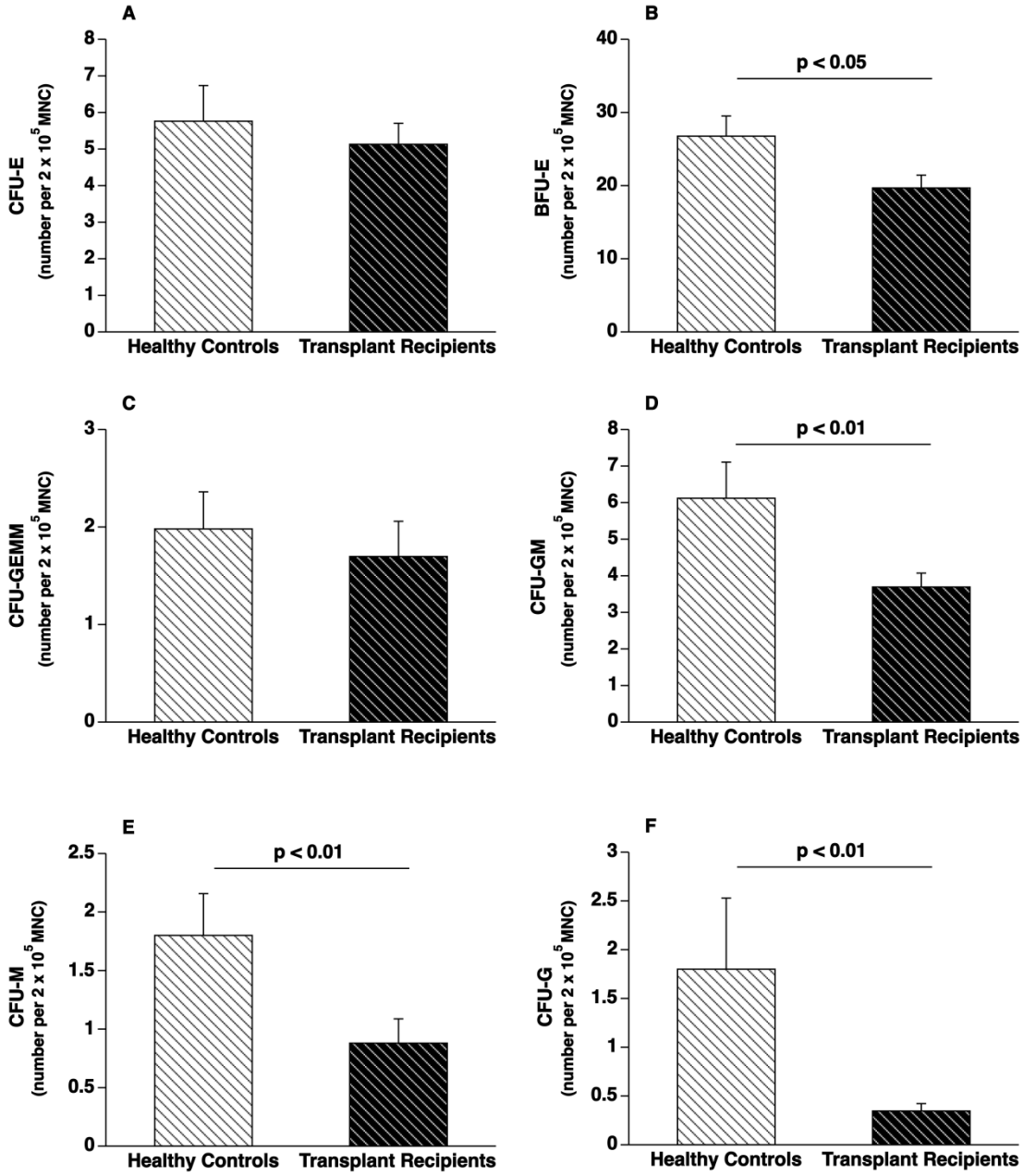

Figure 3.1.3. Enumeration of HPCs in healthy controls and transplant recipients. Comparison of the number of CFU-E (erythroid colony-forming units) (A), BFU-E (erythroid burst-forming units) (B), CFU-GEMM (granulocyte, erythrocyte, macrophage, megakaryocyte colony-forming units) (C), CFU-GM (granulocyte, macrophage colony-forming units) (D), CFU-M (macrophage colony-forming units) (E), and CFU-G (granulocyte colony-forming units) (F) between healthy controls (n=25) and transplant recipients (n=52). MNC: mononuclear cells. All data represent means ± SEM.

3.1.4.4 Circulating apoptotic endothelial cells are significantly greater in patients with CAV than in patients without CAV

The geometric mean of the concentration of circulating endothelial ($CD45^-$ $CD31^{bright}$ $VEGFR\text{-}2^+$) cells, apoptotic endothelial ($CD45^-$ $CD31^{bright}$ $VEGFR\text{-}2^+$ Annexin V positive) cells, and viable endothelial ($CD45^-$ $CD31^{bright}$ $VEGFR\text{-}2^+$ Annexin V negative) cells was increased by 1.7-fold (p=0.062), 1.5-fold (p=NS), and 1.8-fold (p=0.053), respectively, in transplant recipients compared to healthy controls. (Figure 3.1.4). The geometric mean of total, apoptotic, and viable endothelial cells was 2.0-fold (p=0.063), 2.6-fold (p<0.01), and 1.9-fold (p=NS) greater, respectively, in patients with CAV compared to patients without CAV (Table 3.1.3). Gating strategy and flow cytometry analysis of apoptotic CECs are illustrated in Figure 3.1.5.

3.1.4.5 Circulating apoptotic endothelial microparticles are significantly higher in patients with CAV than in patients without CAV

The concentration of circulating endothelial ($CD42a^-$ $CD144^+$) microparticles, apoptotic endothelial ($CD42a^-$ $CD144^+$ Annexin V positive) microparticles, and viable endothelial ($CD42a^-$ $CD144^+$ Annexin V negative) microparticles was 2.6-fold (p<0.0001), 2.0-fold (p<0.001), and 2.6-fold (p<0.0001) greater, respectively, in transplant recipients compared to healthy controls (Figure 3.1.6). The concentration of total, apoptotic, and viable endothelial microparticles was 1.8-fold (p<0.05), 2.0-fold (p<0.01), and 1.7-fold (p<0.05) greater, respectively, in patients with CAV compared to patients without CAV (Table 3.1.3). Gating strategy and flow cytometry analysis of apoptotic endothelial microparticles are illustrated in Figure 3.1.7.

Table 3.1.3. Comparison of CECs and circulating endothelial microparticles between CAV negative and CAV positive patients.

	Patients without CAV (n=22)	Patients with CAV (n=30)	P value
Total CECs (ln (number/µl blood))	-0.268 ± 0.266	0.429 ± 0.251	0.0627
Apoptotic CECs (ln (number/µl blood))	-2.07 ± 0.25	-1.11 ± 0.26	0.0099
Viable CECs (ln (number/µl blood))	-0.502 ± 0.281	0.114 ± 0.256	0.111
Total CEMPs (number/µl plasma)	41.4 ± 9.96	72.7 ± 11.0	0.0400
Apoptotic CEMPs (number/µl plasma)	4.06 ± 0.52	8.02 ± 1.18	0.0039
Viable CEMPs (number/µl plasma)	37.3 ± 9.60	64.7 ± 10.6	0.0410

CEMPs: circulating endothelial microparticles. Data on CECs represent means ± SEM of natural logarithm (ln) transformed values. Data on CEMPs represent means ± SEM.

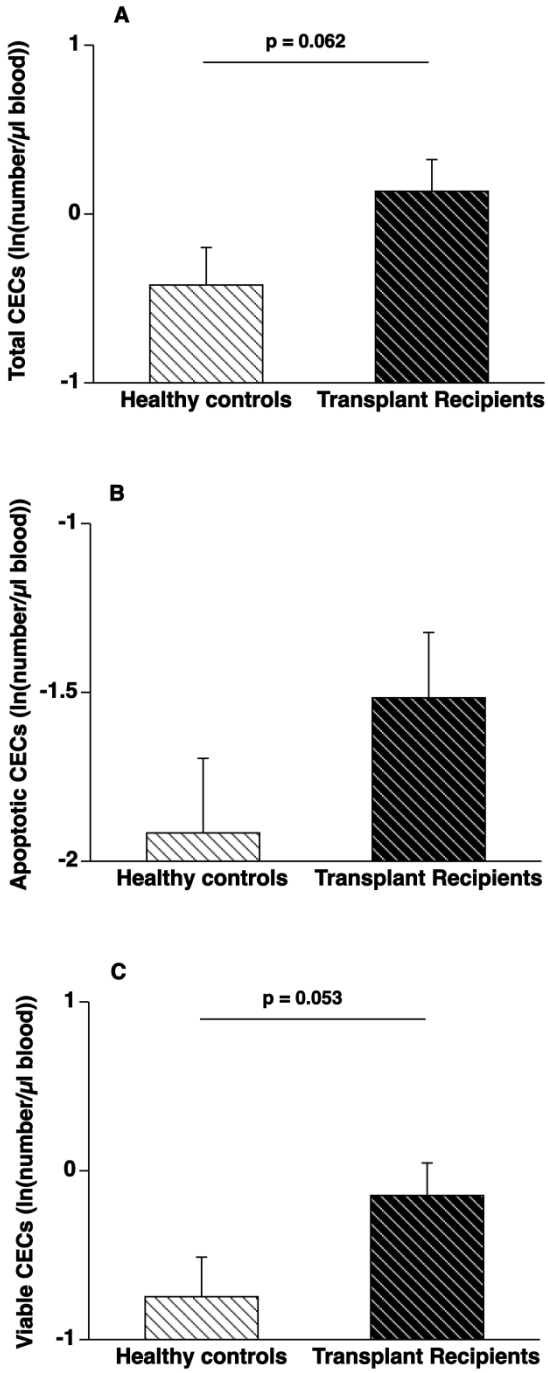

Figure 3.1.4. Quantification of circulating endothelial cells (CECs) by flow cytometry in healthy controls and transplant recipients. Circulating endothelial cells (CECs) (A), apoptotic CECs (B), and viable CECs (C) were compared between healthy controls (n=25) and transplant recipients (n=52). All data represent means ± SEM of natural logarithm (ln) transformed values.

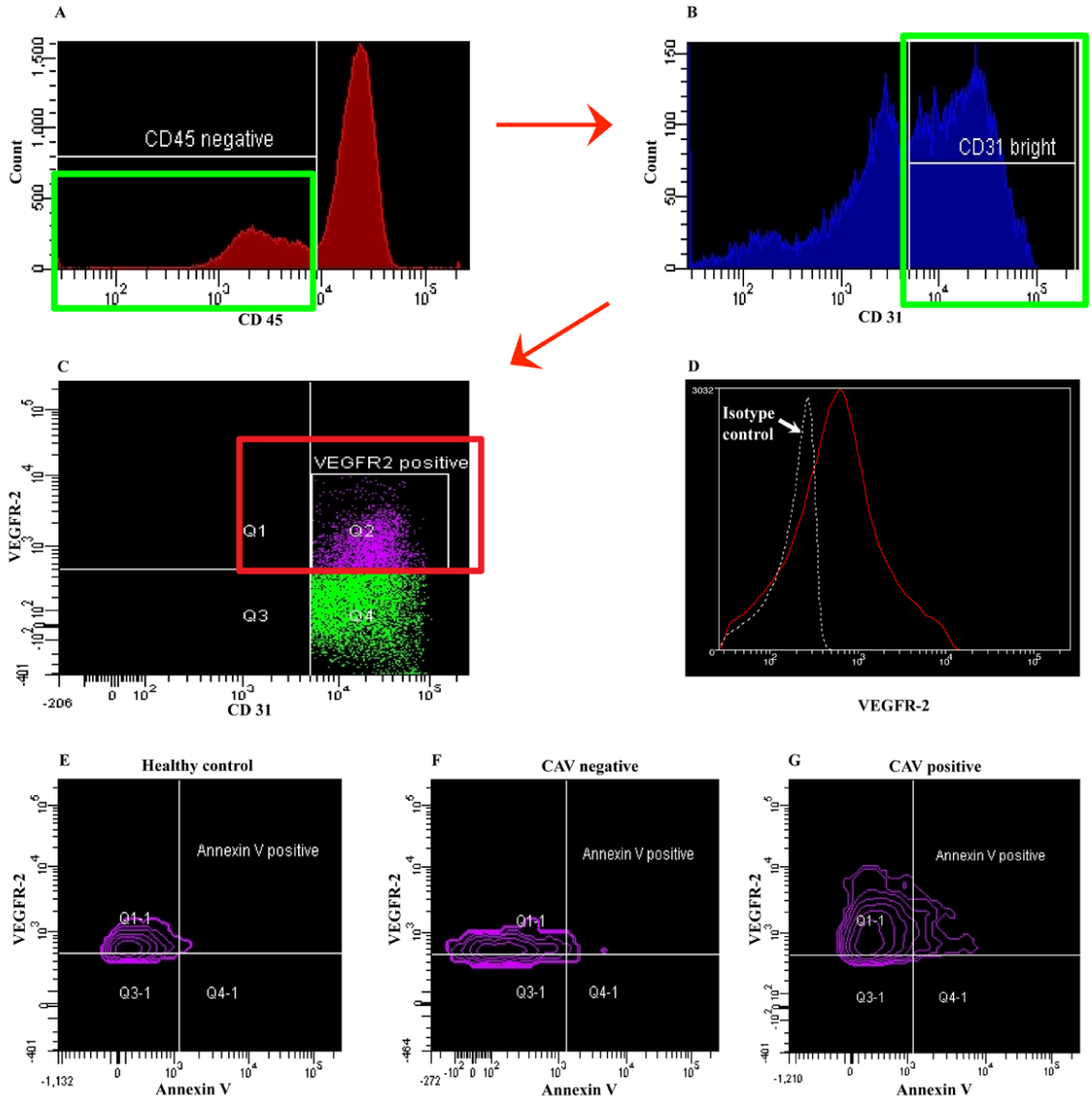

Figure 3.1.5. Flow cytometry analysis of CECs. Gating strategy for CECs, identified as $CD45^-$ $CD31^{bright}$ $VEGFR\text{-}2^+$ mononuclear cells in whole blood samples (A, B, C). An initial gate is set on $CD45^-$ cells out of the mononuclear cellular events (A). Next, a second gate is set on $CD45^-$ $CD31^{bright}$ cells (B), which is further displayed on a FACS scatter plot to identify $CD45^-$ $CD31^{bright}$ $VEGFR\text{-}2^+$ cells (C). The isotype control for VEGFR-2 is shown in panel D. Representative contour plots of Annexin V positive circulating endothelial cells in healthy controls, CAV negative transplant recipients, and CAV positive transplant recipients are shown in panels E, F, and G, respectively.

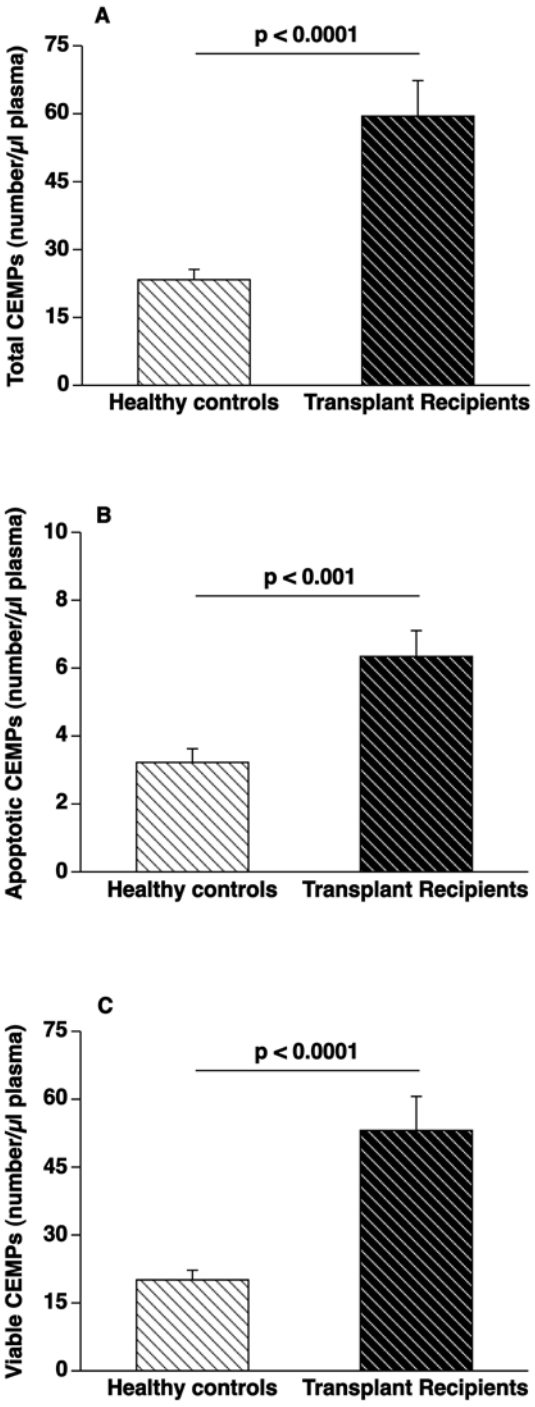

Figure 3.1.6. **Determination of circulating endothelial microparticles by flow cytometry in healthy controls and transplant recipients**. Circulating endothelial microparticles (CEMPs) (A), apoptotic CEMPs (B), and viable CEMPs (C) were analysed in healthy controls (n=25) and transplant recipients (n=52). All data represent means ± SEM.

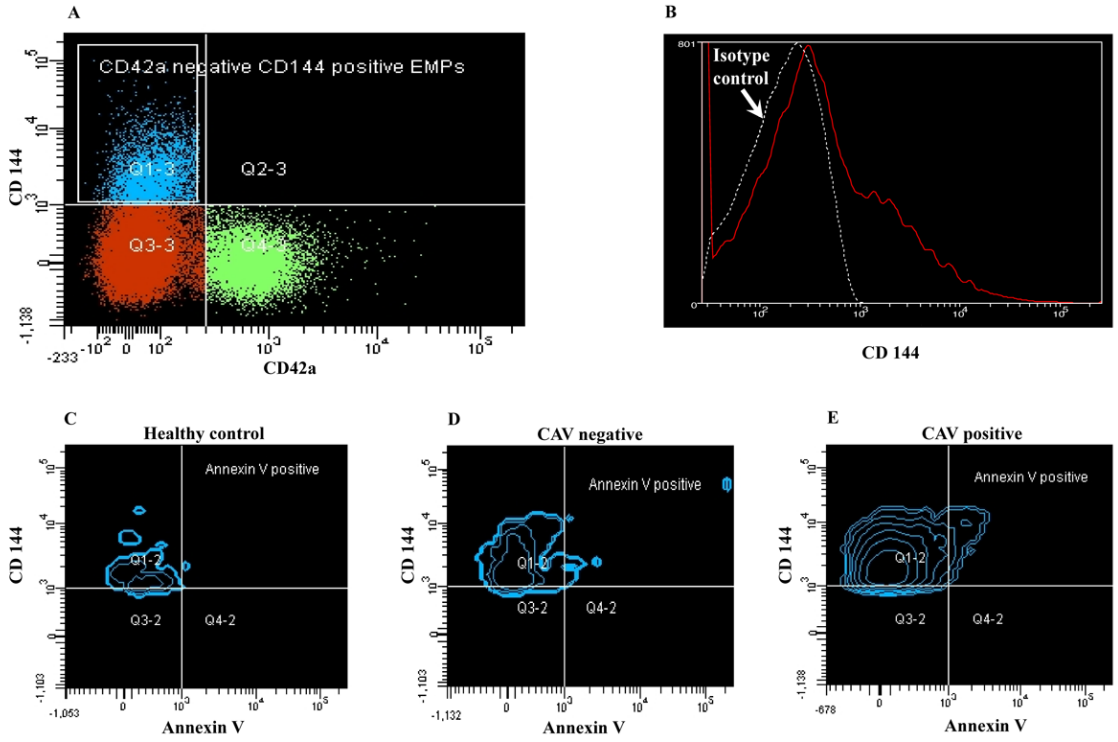

Figure 3.1.7. Flow cytometry analysis of circulating endothelial microparticles. Gating strategy for endothelial microparticles in human plasma, identified as $CD42a^-$ $CD144^+$ microparticles (A). The isotype control for CD144 is shown in panel B. Representative contour plots for Annexin V positive endothelial microparticles in healthy controls, transplant recipients without CAV, and transplant recipients with CAV are shown in panels C, D, and E, respectively.

3.1.4.6 Discrimination between CAV-positive and CAV-negative transplant recipients based on logistic regression and receiver-operating characteristic analysis

Table 3.1.4 summarizes c-statistic values of univariable models. The odds ratio per standard deviation increase of apoptotic CECs (natural logarithm transformed) and of apoptotic endothelial microparticles was 2.32 (95% CI: 1.14-4.71; p=0.0196) and 3.24 (1.17 to 8.96; p=0.0234), respectively. The receiver-operating characteristic curve for the logistic regression function containing apoptotic CECs and apoptotic endothelial microparticles as independent predictors is shown in Figure 3.1.8. The c-statistic was 0.812 (95% CI: 0.692 to 0.932).

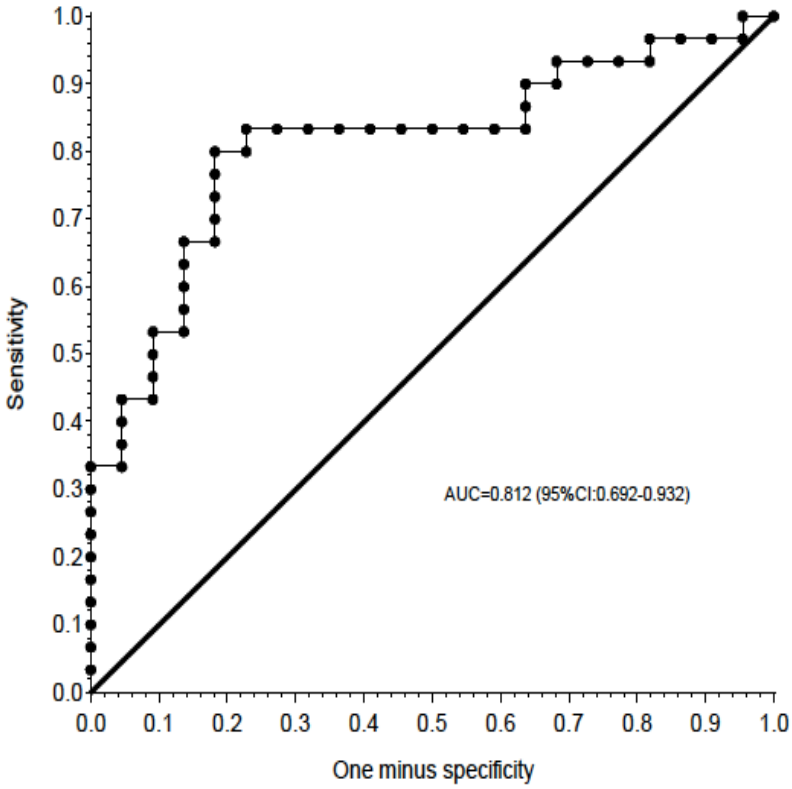

Figure 3.1.8. Receiver-operating characteristic curve for the logistic regression model with apoptotic CECs and apoptotic endothelial microparticles as predictors. This receiver operating characteristic curve illustrates the discriminative ability of the two new biomarkers investigated in the current study. The area under this curve is equal to the c-statistic. AUC = area under the curve; CEC = circulating endothelial cell; CI = confidence interval.

In a logistic regression model with recipient age and creatinine as covariates, apoptotic CECs and apoptotic endothelial microparticles added significant information (chi-square=18.4; df=2; p=0.0001) and were both independent predictors (Table 3.1.5). The c-statistic corresponding to this model was 0.855 (95% CI: 0.756 to 0.953). When recipient age in these models was substituted by time after heart transplantation or age of the transplanted heart, results were essentially unaltered (data not shown).

When steroid dose was introduced in the model, apoptotic CECs and apoptotic endothelial microparticles remained independent predictors (data not shown). The c-statistic corresponding to this model was 0.926 (95% CI: 0.851 to 1.00). There was significant added value of introduction of apoptotic CECs together with endothelial apoptotic microparticles in the model with age, creatinine, and steroid dose (chi-square =16.3; df=2; p=0.0003).

Table 3.1.4. Discrimination between CAV-positive and CAV-negative transplant recipients based on receiver operating characteristic (ROC) analysis.

	c-statistic
Age	0.689 (0.537-0.842)
Creatinine	0.703 (0.552-0.854)
LDL cholesterol	0.660 (0.507-0.813)
Steroid dose	0.758 (0.642-0.873)
CFU-G	0.681 (0.547-0.815)
Apoptotic CECs	0.709 (0.567-0.851)
Apoptotic CEMPs	0.697 (0.554-0.840)

The c-statistic corresponds to the area under the ROC curve. The 95% confidence interval is indicated between brackets. CEC: circulating edothelial cell. CEMP: circulating endothelial microparticle.

Table 3.1.5. Logistic regression model for prediction of CAV.

	OR	**P value**
Age (years)	1.38 (0.66-2.88)	0.399
Creatinine (mg/dL)	3.50 (1.27-9.66)	0.0158
Apoptotic CECs (ln number/µl)	4.34 (1.40-13.5)	0.0112
Apoptotic CEMPs (number/100 µl)	5.30 (1.40-20.0)	0.0141

The 95% confidence interval is indicated between brackets. Odds ratios (OR) are expressed per standard deviation increase. CEC: circulating edothelial cell. CEMP: circulating endothelial microparticle.

3.1.5 DISCUSSION

This is the first study to demonstrate that apoptotic CECs and apoptotic endothelial microparticles are independent predictors of CAV and that the combination of these two biomarkers has a high discriminative ability between patients without CAV and with CAV.

3.1.5.1 Clinical parameters and CAV

A salient finding of the current study is that steroid use was more prevalent in CAV-positive patients than in CAV-negative patients. This is in line with a report of the Stanford group in 2004[23] in a population of patients on an immunosuppressive background therapy predominantly comprising cyclosporine and azathioprine. Our observation cannot be explained by a continuation of steroids following severe acute rejection episodes in the first year, since the proportion of patients with severe acute rejection episodes in the first year was similar in patients with CAV compared to patients without CAV. Second, difference of steroid use can also not be the result of tailoring of therapy according to renal function since creatinine levels were not different between steroid users and non-steroid users (data not shown). On the other hand, the time after heart transplantation was significantly ($p<0.05$) longer in patients on steroids (11.6 ± 0.9 years) compared to non-steroid users (9.1 ± 0.5 years). This reflects an evolution to steroid free immunosuppressive regimens in more recently transplanted patients. However, steroid use was also predictive of the presence of CAV in models in which the time after transplantation was introduced as a covariate. Therefore, we cannot exclude the possibility that continued steroid use plays a causative role in the development of CAV.

Impaired renal function was an independent predictor of the presence of CAV. The natural history of renal function following heart transplantation is characterised by a decline in renal function[24]. Age, pre-transplant glomerular filtration rate, pre-transplant diabetes, and pre-transplant hypertension are important risk factors for a decrement of renal function[24]. Nephrotoxicity of calcineurin inhibitors is largely responsible for the progressive development of renal dysfunction[25,26]. Our results are in line with those of Schober *et al*.[27]. However, impaired renal function was not a predictor in a study with angiographic follow-up limited to 4 years[28].

The observation that time after transplantation does not predict the CAV status may seem illogical. However, the recruitment of patients in the current study was restricted to heart transplant recipients between 5 and 15 years after transplantation undergoing coronary angiography during follow-up. Because of this selection procedure, time after transplantation tends to be similar in the CAV-positive and CAV-negative patients.

3.1.5.2 Biomarkers of endothelial repair and CAV

We did not observe a difference between CAV-positive and CAV-negative patients in the number of EPCs quantified as the number of $CD34^+$ $VEGFR\text{-}2^+$ cells by flow cytometry analysis or as the number of Dil-acLDL and FITC-labeled UEA-1 lectin double positive cells after 4 days of *ex vivo* culture. Furthermore, EPC function was similar in patients with CAV and without CAV. In contrast, Simper and colleagues[29] observed that the number of EPC colony-forming units (late outgrowth EPCs) appearing over a 6-week culture period was significantly lower in 8 patients with CAV compared to 7 patients without CAV. However, our results are in line with those of Thomas *et al*.[30] and Schober *et al*.[27] who quantified EPC count as the number of $CD34^+$ $VEGFR\text{-}2^+$ cells. Interestingly, Schober *et al*.[27] demonstrated that the number of $CD34^+$ $CD140b^+$ smooth muscle progenitor cells is independently associated with the presence of CAV.

EPC number based on *ex vivo* culture assay was lower and EPC function was impaired in transplant recipients compared to healthy controls. Different classes of immunosuppressive drugs may affect EPC biology[31,32]. Therefore, the lack of discriminative ability of EPC number and EPC function to detect the presence of CAV may be related to a generalized impairment of these parameters in heart transplant recipients receiving different classes of immunosuppressive drugs. In contrast, in a murine model of transplant vasculopathy without immunosuppression, increased EPC number and enhanced EPC function attenuated progression of the disease[33,34].

Because the term EPC is used in a very broad sense in the literature, EPCs encompass different categories of cells with different phenotypic and functional properties that affect neovascularization and reendothelialization. A salient observation in the current study is that EPC number defined as $CD34^+$ $VEGFR\text{-}2^+$ cells did not differ between healthy controls and transplant recipients in contrast to EPC number determined by *ex vivo* cell culture. This basically reflects that the same term is used for entirely different categories of cells. Many so-called EPCs are in fact hematopoietic lineage cells. Hematopoietic progenitor cells may differentiate into proangiogenic cells[12] and may promote neovascularization and endothelial repair in a paracrine way. HPC culture assays showed a significantly lower number of granulocyte colony-forming units in the peripheral blood of CAV-positive compared to CAV-negative patients. However, in multivariate logistic regression, the number of granulocyte colony-forming units was not an independent predictor of the presence of CAV (data not shown). Taken together, biomarkers of endothelial repair that were evaluated in the current study are not suited for clinical prediction models of CAV. However, we cannot exclude that other assays of endothelial repair discriminate between CAV-negative and CAV-positive patients in multivariable models.

3.1.5.3 Biomarkers of endothelial injury and CAV

The concentration of apoptotic endothelial microparticles and of apoptotic CECs was significantly different between CAV-positive and CAV-negative patients. As indicated by the c-statistic value of

0.812, the logistic regression model combining these two biomarkers provides high discriminative ability between CAV-positive and CAV-negative patients.
In several logistic regression models, the introduction of apoptotic CECs and apoptotic endothelial microparticles consistently resulted in added value, indicating that these biomarkers are robust independent predictors. Whereas the final model in the current study is restricted to age, creatinine, apoptotic CECs, and apoptotic endothelial microparticles, the latter two parameters remain significant predictors in models with creatinine and steroid dose. Importantly, the discriminative ability of these two biomarkers was also preserved in models in which recipient age was replaced by time after transplantation or by age of the transplanted heart.

3.1.5.4 Study limitations

Because the number of subjects in the current study is limited to 22 CAV-negative patients and 30 CAV-positive patients, models with more than 3 predictors should be interpreted with caution[35]. Inclusion of too many predictors leads to overfitting of the data and C-indices are overestimated[35]. However, the additional models strongly suggest that the discriminative ability of apoptotic endothelial microparticles and apoptotic CECs is not affected by potential confounders such as age, renal function, and steroid dose.
Annexin V positivity, corresponding to phosphatidylserine externalization, should be interpreted with caution. It is already positive in an early and potentially reversible stage of apoptosis[36,37] and is not entirely specific for apoptosis[38,39]. However, these issues do not undermine the discriminative ability of Annexin V positive categories that were evaluated in the current study.

3.1.5.5 Future studies

The high discriminative ability of apoptotic CECs and apoptotic endothelial microparticles provides a solid foundation for the further development of clinical prediction models of CAV in the framework of prospective studies that evaluate CAV by coronary intravascular ultrasound. Prospective prediction models may lead to a more rational and more tailored use of coronary angiography and intravascular ultrasound that are not without risk. In addition, risk prediction models may allow quicker intervention and may help in the design of new randomized clinical trials to optimize therapy in heart transplant recipients.

3.1.6 CONCLUSIONS

Apoptotic CECs and apoptotic endothelial microparticles predict the presence of CAV independent of the age of the recipient, age of the transplanted heart, creatinine level, steroid use, and number of granulocyte colony-forming units. The results of the current study are compatible with the hypothesis that endothelial activation and injury are involved in the development of CAV.

3.1.7 REFERENCES

1. Schmauss, D. ,Weis, M. Cardiac allograft vasculopathy: recent developments. *Circulation* **117**, 2131-2141 (2008).
2. Rahmani, M., Cruz, R.P., Granville, D.J. ,McManus, B.M. Allograft vasculopathy versus atherosclerosis. *Circulation research* **99**, 801-815 (2006).
3. Stehlik, J., Edwards, L.B., Kucheryavaya, A.Y., Benden, C., Christie, J.D., Dobbels, F., Kirk, R., Rahmel, A.O. ,Hertz, M.I. The Registry of the International Society for Heart and Lung Transplantation: Twenty-eighth Adult Heart Transplant Report--2011. *J Heart Lung Transplant* **30**, 1078-1094 (2011).
4. Hunt, S.A. ,Haddad, F. The changing face of heart transplantation. *J Am Coll Cardiol* **52**, 587-598 (2008).
5. Vassalli, G., Gallino, A., Weis, M., von Scheidt, W., Kappenberger, L., von Segesser, L.K. ,Goy, J.J. Alloimmunity and nonimmunologic risk factors in cardiac allograft vasculopathy. *Eur Heart J* **24**, 1180-1188 (2003).
6. Woywodt, A., Bahlmann, F.H., De Groot, K., Haller, H. ,Haubitz, M. Circulating endothelial cells: life, death, detachment and repair of the endothelial cell layer. *Nephrol Dial Transplant* **17**, 1728-1730 (2002).
7. van Engeland, M., Nieland, L.J., Ramaekers, F.C., Schutte, B. ,Reutelingsperger, C.P. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* **31**, 1-9 (1998).
8. Piccin, A., Murphy, W.G. ,Smith, O.P. Circulating microparticles: pathophysiology and clinical implications. *Blood Rev* **21**, 157-171 (2007).
9. Jimenez, J.J., Jy, W., Mauro, L.M., Soderland, C., Horstman, L.L. ,Ahn, Y.S. Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. *Thromb Res* **109**, 175-180 (2003).
10. Faure, V., Dou, L., Sabatier, F., Cerini, C., Sampol, J., Berland, Y., Brunet, P. ,Dignat-George, F. Elevation of circulating endothelial microparticles in patients with chronic renal failure. *J Thromb Haemost* **4**, 566-573 (2006).
11. Hirschi, K.K., Ingram, D.A. ,Yoder, M.C. Assessing identity, phenotype, and fate of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* **28**, 1584-1595 (2008).
12. Wara, A.K., Croce, K., Foo, S., Sun, X., Icli, B., Tesmenitsky, Y., Esen, F., Rosenzweig, A. ,Feinberg, M.W. Bone marrow-derived CMPs and GMPs represent highly functional pro-angiogenic cells: implications for ischemic cardiovascular disease. *Blood* (2011).
13. Mehra, M.R., Crespo-Leiro, M.G., Dipchand, A., Ensminger, S.M., Hiemann, N.E., Kobashigawa, J.A., Madsen, J., Parameshwar, J., Starling, R.C. ,Uber, P.A. International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for cardiac allograft vasculopathy-2010. *J Heart Lung Transplant* **29**, 717-727 (2010).

14. Billingham, M.E., Cary, N.R., Hammond, M.E., Kemnitz, J., Marboe, C., McCallister, H.A., Snovar, D.C., Winters, G.L. ,Zerbe, A. A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. The International Society for Heart Transplantation. *J Heart Transplant* **9**, 587-593 (1990).
15. Mehra, M.R., Ventura, H.O., Chambers, R.B., Ramireddy, K., Smart, F.W. ,Stapleton, D.D. The prognostic impact of immunosuppression and cellular rejection on cardiac allograft vasculopathy: time for a reappraisal. *J Heart Lung Transplant* **16**, 743-751 (1997).
16. Vasa, M., Fichtlscherer, S., Aicher, A., Adler, K., Urbich, C., Martin, H., Zeiher, A.M. ,Dimmeler, S. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circulation research* **89**, E1-7 (2001).
17. Honold, J., Lehmann, R., Heeschen, C., Walter, D.H., Assmus, B., Sasaki, K., Martin, H., Haendeler, J., Zeiher, A.M. ,Dimmeler, S. Effects of granulocyte colony simulating factor on functional activities of endothelial progenitor cells in patients with chronic ischemic heart disease. *Arterioscler Thromb Vasc Biol* **26**, 2238-2243 (2006).
18. Vasa, M., Fichtlscherer, S., Adler, K., Aicher, A., Martin, H., Zeiher, A.M. ,Dimmeler, S. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation* **103**, 2885-2890 (2001).
19. Schmidt-Lucke, C., Rossig, L., Fichtlscherer, S., Vasa, M., Britten, M., Kamper, U., Dimmeler, S. ,Zeiher, A.M. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. *Circulation* **111**, 2981-2987 (2005).
20. Duda, D.G., Cohen, K.S., Scadden, D.T. ,Jain, R.K. A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. *Nat Protoc* **2**, 805-810 (2007).
21. Schmidt-Lucke, C., Fichtlscherer, S., Rossig, L., Kamper, U. ,Dimmeler, S. Improvement of endothelial damage and regeneration indexes in patients with coronary artery disease after 4 weeks of statin therapy. *Atherosclerosis* **211**, 249-254 (2010).
22. Koga, H., Sugiyama, S., Kugiyama, K., Watanabe, K., Fukushima, H., Tanaka, T., Sakamoto, T., Yoshimura, M., Jinnouchi, H. ,Ogawa, H. Elevated levels of VE-cadherin-positive endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery disease. *J Am Coll Cardiol* **45**, 1622-1630 (2005).
23. Shiba, N., Chan, M.C., Kwok, B.W., Valantine, H.A., Robbins, R.C. ,Hunt, S.A. Analysis of survivors more than 10 years after heart transplantation in the cyclosporine era: Stanford experience. *J Heart Lung Transplant* **23**, 155-164 (2004).
24. Al Aly, Z., Abbas, S., Moore, E., Diallo, O., Hauptman, P.J. ,Bastani, B. The natural history of renal function following orthotopic heart transplant. *Clin Transplant* **19**, 683-689 (2005).
25. Groetzner, J., Kaczmarek, I., Schulz, U., Stegemann, E., Kaiser, K., Wittwer, T., Schirmer, J., Voss, M., Strauch, J., Wahlers, T., Sohn, H.Y., Wagner, F., Tenderich, G., Stempfle, H.U., Mueller-Ehmsen, J., Schmid, C., Vogeser, M., Koch, K.C., Reichenspurner, H.,

Daebritz, S., Meiser, B. ,Reichart, B. Mycophenolate and sirolimus as calcineurin inhibitor-free immunosuppression improves renal function better than calcineurin inhibitor-reduction in late cardiac transplant recipients with chronic renal failure. *Transplantation* **87**, 726-733 (2009).

26. Flechner, S.M., Kobashigawa, J. ,Klintmalm, G. Calcineurin inhibitor-sparing regimens in solid organ transplantation: focus on improving renal function and nephrotoxicity. *Clin Transplant* **22**, 1-15 (2008).
27. Schober, A., Hristov, M., Kofler, S., Forbrig, R., Lohr, B., Heussen, N., Zhe, Z., Akhtar, S., Schumann, U., Krotz, F., Leibig, M., Konig, A., Kaczmarek, I., Reichart, B., Klauss, V., Weber, C. ,Sohn, H.Y. CD34+CD140b+ cells and circulating CXCL12 correlate with the angiographically assessed severity of cardiac allograft vasculopathy. *Eur Heart J* **32**, 476-484 (2011).
28. Arora, S., Andreassen, A., Simonsen, S., Gude, E., Dahl, C., Skaardal, R., Hoel, I., Geiran, O. ,Gullestad, L. Prognostic importance of renal function 1 year after heart transplantation for all-cause and cardiac mortality and development of allograft vasculopathy. *Transplantation* **84**, 149-154 (2007).
29. Simper, D., Wang, S., Deb, A., Holmes, D., McGregor, C., Frantz, R., Kushwaha, S.S. ,Caplice, N.M. Endothelial progenitor cells are decreased in blood of cardiac allograft patients with vasculopathy and endothelial cells of noncardiac origin are enriched in transplant atherosclerosis. *Circulation* **108**, 143-149 (2003).
30. Thomas, H.E., Parry, G., Dark, J.H., Arthur, H.M. ,Keavney, B.D. Circulating endothelial progenitor cell numbers are not associated with donor organ age or allograft vasculopathy in cardiac transplant recipients. *Atherosclerosis* **202**, 612-616 (2009).
31. Davies, W.R., Wang, S., Oi, K., Bailey, K.R., Tazelaar, H.D., Caplice, N.M. ,McGregor, C.G. Cyclosporine decreases vascular progenitor cell numbers after cardiac transplantation and attenuates progenitor cell growth in vitro. *J Heart Lung Transplant* **24**, 1868-1877 (2005).
32. Sathya, C.J., Sheshgiri, R., Prodger, J., Tumiati, L., Delgado, D., Ross, H.J. ,Rao, V. Correlation between circulating endothelial progenitor cell function and allograft rejection in heart transplant patients. *Transpl Int* **23**, 641-648 (2010).
33. Feng, Y., Jacobs, F., Van Craeyveld, E., Brunaud, C., Snoeys, J., Tjwa, M., Van Linthout, S. ,De Geest, B. Human ApoA-I transfer attenuates transplant arteriosclerosis via enhanced incorporation of bone marrow-derived endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* **28**, 278-283 (2008).
34. Feng, Y., van Eck, M., Van Craeyveld, E., Jacobs, F., Carlier, V., Van Linthout, S., Erdel, M., Tjwa, M. ,De Geest, B. Critical role of scavenger receptor-BI-expressing bone marrow-derived endothelial progenitor cells in the attenuation of allograft vasculopathy after human apo A-I transfer. *Blood* **113**, 755-764 (2009).

35. Peduzzi, P., Concato, J., Kemper, E., Holford, T.R. ,Feinstein, A.R. A simulation study of the number of events per variable in logistic regression analysis. *J Clin Epidemiol* **49**, 1373-1379 (1996).
36. Geske, F.J., Lieberman, R., Strange, R. ,Gerschenson, L.E. Early stages of p53-induced apoptosis are reversible. *Cell Death Differ* **8**, 182-191 (2001).
37. Hammill, A.K., Uhr, J.W. ,Scheuermann, R.H. Annexin V staining due to loss of membrane asymmetry can be reversible and precede commitment to apoptotic death. *Exp Cell Res* **251**, 16-21 (1999).
38. Balasubramanian, K., Mirnikjoo, B. ,Schroit, A.J. Regulated externalization of phosphatidylserine at the cell surface: implications for apoptosis. *J Biol Chem* **282**, 18357-18364 (2007).
39. Smrz, D., Draberova, L. ,Draber, P. Non-apoptotic phosphatidylserine externalization induced by engagement of glycosylphosphatidylinositol-anchored proteins. *J Biol Chem* **282**, 10487-10497 (2007).

3.2 MARKERS OF ENDOTHELIAL INJURY AND PLATELET MICROPARTICLES ARE DISTINCT IN PATIENTS WITH STABLE NATIVE CORONARY ARTERY DISEASE AND WITH CARDIAC ALLOGRAFT VASCULOPATHY*

3.2.1 ABSTRACT

Background: Endothelial injury plays a key role in both native coronary artery disease (CAD) and cardiac allograft vasculopathy (CAV). Cellular biomarkers of endothelial injury (circulating endothelial microparticles (CEMPs) and circulating endothelial cells (CECs)) may discriminate between endothelial activation and irreversible endothelial damage. The hypothesis that endothelial injury and circulating platelet microparticles (CPMPs) are distinct in both types of arteriosclerosis was investigated.

Methods and Results: The geometric mean of the concentration of CECs ($CD45^-$ $CD31^{bright}$ $VEGFR\text{-}2^+$) was 2.90-fold ($p<0.001$) and 2.34-fold ($p<0.05$) higher in patients with stable native CAD (n=80) and with CAV (n=30), respectively, compared to healthy controls (n=25). No significant difference in total, Annexin V negative, and Annexin V positive (apoptotic) CECs was observed between patients with native CAD and with CAV. The concentration of Annexin V negative CEMPs ($CD144^+$ $CD42a^-$) was 59.2% ($p<0.01$) higher in transplant recipients with CAV than in native CAD patients but no difference in Annexin V positive CEMPs was observed. The median value of total $CD61^+$ CPMPs in native CAD patients was 69.4% ($p<0.001$) and 71.6% ($p<0.001$) lower compared to healthy controls and transplant recipients with CAV, respectively. These differences were even more pronounced when $CD42a^+CD31^+$ CPMPs were quantified.

Conclusion: The selective increase of Annexin V negative CEMPs and the absence of a difference in Annexin V positive CECs strongly suggest increased endothelial activation but not endothelial apoptosis in CAV positive patients compared to stable CAD patients. Use of antiplatelet drugs likely underlies the strikingly lower levels of CPMPs in patients with native CAD.

3.2.2 INTRODUCTION

Cardiac allograft vasculopathy (CAV) is a particular type of arteriosclerosis with many similarities but also significant differences compared to native coronary artery disease (CAD). Atherosclerosis in patients with stable native CAD is characterized by the presence of atheromata that contain a lipid core filled with extracellular cholesterol and cellular debris and are covered by a fibrous cap. In contrast, fibromuscular intimal hyperplasia is the most prominent lesion type of CAV and mainly consists of smooth muscle cells and extracellular matrix[1]. It tends to be circumferential and not

* This chapter has been published as: **Singh, N.**, Vanhaecke, J., Van Cleemput, J., De Geest, B.. Markers of endothelial injury and platelet microparticles are distinct in patients with stable native coronary artery disease and with cardiac allograft vasculopathy. *International Journal of Cardiology* 2015; 179, 331-333

only involves the proximal part of epicardial arteries but also smaller epicardial arteries and intramyocardial arteries.

Endothelial injury is assumed to play a key role in the initiation and progression of both native CAD and CAV[2, 3]. In the response-to-injury hypothesis of atherosclerosis of Ross and Glomset, endothelial injury was originally defined as endothelial denudation resulting from focal desquamation of endothelium[4, 5]. Later versions of the response-to-injury hypothesis emphasized endothelial dysfunction rather than denudation[2, 6]. Biochemical surrogate biomarkers of endothelial injury (e.g. von Willebrand factor, soluble thrombomodulin, soluble vascular cell adhesion molecule, soluble intercellular adhesion molecule, and soluble E-selectin) do not discriminate between endothelial activation and irreversible endothelial damage and lack endothelial specificity[7]. In contrast, cellular biomarkers of endothelial injury (circulating endothelial microparticles (CEMPs) and circulating endothelial cells (CECs)) are endothelial-specific. Microparticles are 0.1 µm to 1 µm large membrane vesicles that are released following cell activation or apoptosis[8]. Levels of CEMPs inversely correlate with the amplitude of flow-mediated dilatation[9-12] indicating that they are a valid biomarker of endothelial function. In contrast to CEMPs, CECs constitute a parameter of irreversible damage of the endothelium[13-15]. CECs are mature endothelial cells that originate by detachment from the endothelial monolayer as a result of an endothelial insult. Thus, by combined quantification of CEMPs and CECs, it is possible to evaluate to which extent endothelial injury represents endothelial activation or endothelial denudation. Since the lesions of native CAD and CAV are clearly different, the primary hypothesis investigated in the current study was whether a distinct pattern of endothelial injury (endothelial activation versus endothelial denudation) would be observed in these two types of arteriosclerosis.

Platelets can adhere to dysfunctional endothelium, exposed collagen, and macrophages, and promote initiation and progression of atherosclerosis[2, 5]. Platelet microparticles are a marker of platelet activation. The secondary objective of the current study was to compare circulating platelet microparticles (CPMPs) in patients with native CAD and with CAV.

3.2.3 METHODS

3.2.3.1 Study design

Eighty patients with clinically stable native coronary artery disease (CAD) and thirty heart transplant recipients with cardiac allograft vasculopathy (CAV) were recruited in the current cross-sectional study. Stable native CAD patients were defined by the presence of at least one stenosis of 50% or more demonstrated by diagnostic coronary angiography. CAV was diagnosed in heart transplant recipients undergoing coronary angiography between 5 and 15 years after heart transplantation[16]. CAV was graded according to the International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for CAV-2010[17]. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected by

the *a priori* approval of the protocol by the Ethics Committee of the University Hospital Gasthuisberg. Written informed consent was obtained from all participants. The reference control group included 25 healthy control subjects (12 males and 13 females). Average age of healthy controls was 43.2 ± 2.0 years.

3.2.3.2 Determination of the number of circulating endothelial cells, endothelial microparticles, and platelet microparticles by flow cytometry

Circulating endothelial cells (CECs) were defined as $CD45^-$ $CD31^{bright}$ $VEGFR\text{-}2^+$ mononuclear cells as described by Duda *et al*.[18]. Platelet-poor plasma derived from sodium citrate anticoagulated blood was used for microparticle quantification by flow cytometry analysis. Circulating endothelial microparticles (CEMPs) were defined as CD144 $(VE\text{-}Cadherin)^+$ CD42a $(GPIX)^-$ microparticles as described before[16]. Circulating platelet microparticles (CPMPs) were determined as $CD61^+$ microparticles[19, 20] or alternatively as $CD31^+$ $CD42a^+$ microparticles[21, 22]. Annexin V (Immunotools, Friesoythe, Germany) staining was used to discriminate between apoptotic and non-apoptotic microparticles. For CPMPs quantification, incubation of platelet-poor plasma was performed with mouse monoclonal antibodies directed against human CD61 (Biolegend, San Diego, California, USA), human CD31 (BD Biosciences, San Jose, California, USA), and human CD42a (BD Biosciences, San Jose, California, USA), and with Annexin V. Samples were acquired on a low flow rate (30 µl/min) for 5 minutes using a BD FACSCantoII flow cytometer and BD FACSDIVA software version 1.2.6 (BD Biosciences).

3.2.3.3 Statistical analysis

Clinical and laboratory parameters were compared between stable native CAD patients and CAV positive patients using Instat 3 (Graphpad software, San Diego, CA, USA). Continuous variables were summarized by means, standard error of the mean, and sample size, and were compared between two groups by Student t-test or if indicated a Mann-Whitney test was applied. Fisher's exact test was used to compare the categorical data between stable native CAD patients and CAV positive patients. CECs, CEMPs, and CPMPs were compared between healthy controls, stable native CAD patients, and heart transplant recipients with CAV by analysis of variance followed by Tukey's post hoc test. If indicated, a logarithmic transformation of the data was performed or a Kruskal-Wallis test followed by Dunn's post hoc test was applied. A p-value of less than 0.05 was considered statistically significant.

3.2.4 RESULTS

3.2.4.1 Patient characteristics

Clinical characteristics, laboratory parameters, and medical therapy in patients with stable native CAD (n=80) and heart transplant recipients with CAV (n=30) are summarized in Table 3.2.1. Patients with native CAD were 4.7 years ($p<0.05$) older, had a lower prevalence of hypertension ($p<0.0001$), and a higher body mass index than patients with CAV ($p<0.05$). C-reactive protein levels were 6.32-fold ($p<0.001$) higher in heart transplant recipients with CAV than in patients with native CAD. Lipoprotein levels were very similar and statin use was generalized in both conditions. The use of antiplatelet drugs was generalized in patients with native CAD and was restricted to one heart transplant recipient with CAV.

3.2.4.2 Circulating endothelial cells are increased in stable native CAD patients and heart transplant recipients with CAV to a similar extent

The geometric mean of the concentration of circulating endothelial ($CD45^{-}$ $CD31^{bright}$ $VEGFR\text{-}2^{+}$) cells (CECs) was increased 2.90-fold ($p<0.001$) and 2.34-fold ($p<0.05$) in patients with native CAD and with CAV, respectively, compared to healthy controls (Figure 3.2.1A). The elevation of Annexin V negative CECs and Annexin V positive CECs in patients with native CAD and CAV compared to healthy controls is illustrated in Figure 3.2.1B and 3.2.1C, respectively. No significant differences in any of these parameters were observed between patients with native CAD and transplant recipients with CAV. Taken together, the number of CECs as a parameter of irreversible endothelial damage is similarly increased in both types of arteriosclerosis.

3.2.4.3 Total endothelial microparticles and Annexin V negative endothelial microparticles are higher in transplant recipients with CAV than in patients with native CAD

Circulating endothelial ($CD42a^{-}$ $CD144^{+}$) microparticles (CEMPs), Annexin V negative CEMPs, and Annexin V positive CEMPs were elevated in patients with native CAD and transplant recipients with CAV compared to healthy controls as illustrated in Figure 3.2.2A, Figure 3.2.2B, and Figure 3.2.2C, respectively. The concentration of CEMPs (Figure 3.2.2A) and Annexin V negative CEMPs (Figure 3.2.2B) was 45.8% ($p<0.01$) and 59.2% ($p<0.01$) higher, respectively, in transplant recipients with CAV than in patients with native CAD. No significant difference in Annexin V positive CEMPs was observed between patients with native CAD and CAV (Figure 3.2.2C). Taken together, the selective increase of Annexin V negative CEMPs in transplant recipients with CAV compared to patients with native CAD is compatible with more pronounced endothelial cell activation in the former.

Table 3.2.1. Clinical characteristics, laboratory parameters, and hypolipidemic and antiplatelet therapy in patients with stable native CAD and in transplant recipients with CAV.

	Patients with stable native CAD (n=80)	Patients with CAV (n=30)	P value
Age at inclusion in the study (years)	69.6 ± 1.2	64.9 ± 2.1	0.0459
Sex (male/female)	63 (78.8%)/17 (21.3%)	24 (80.0%)/6 (20.0%)	1.00
Current smoker (%)	7.5%	0%	0.186
Hypertension (%)	47.5%	100%	<0.0001
Diabetes (%)	25.0%	36.7%	0.242
Body mass index (kg/m^2)	27.5 ± 0.6	25.5 ± 0.5	0.0337
Platelet count (10^9/L)	222 ± 5	217 ± 12	0.150
Leukocyte count (10^9/L)	7.19 ± 0.22	6.87 ± 0.24	0.398
Monocyte count (10^9/L)	0.435 ± 0.029	0.313 ± 0.039	0.012
Lymphocyte count (10^9/L)	1.94 ± 0.08	1.28 ± 0.12	<0.0001
Neutrophil count (10^9/L)	4.22 ± 0.20	3.78 ± 0.36	0.213
Creatinine (mg/dl)	1.05 ± 0.03	1.60 ± 0.08	<0.0001
CRP (mg/l)	1.95 ± 0.18	12.4 ± 3.3	<0.0001
Cholesterol (mg/dl)	160 ± 4	156 ± 6	0.548
Triglycerides (mg/dl)	132 ± 7	125 ± 12	0.557
HDL cholesterol (mg/dl)	50.2 ± 1.8	54.2 ± 3.4	0.353
LDL cholesterol (mg/dl)	83.6 ± 3.0	77.1 ± 4.0	0.402
Acetylsalicylic acid (%)	88.8%	3.33%	<0.0001
Statins (%)	95.0%	100%	0.573
Clopidogrel (%)	13.8%	0%	0.0334

Data are expressed as means ± SEM for continuous variables.

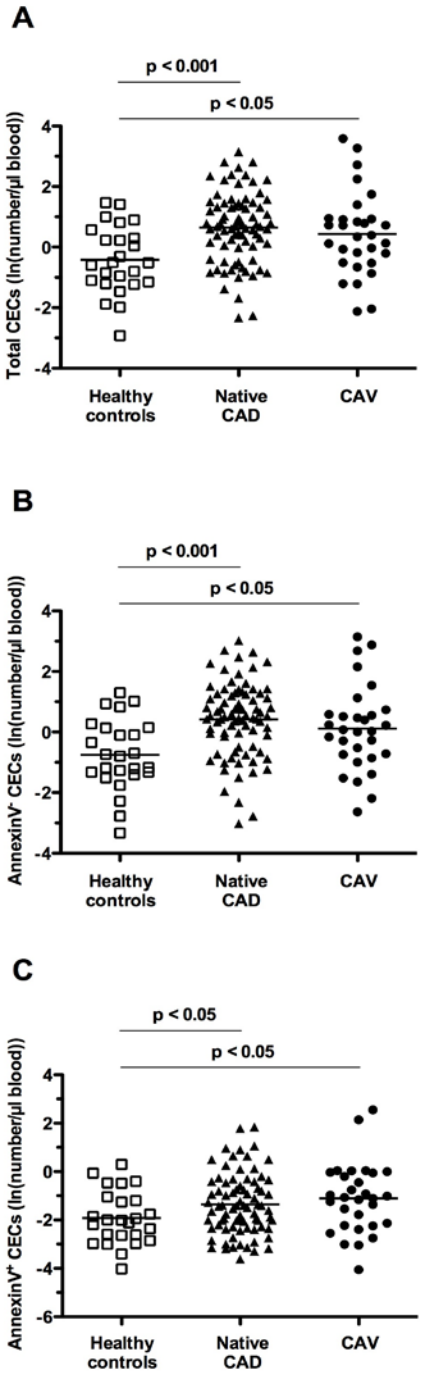

Figure 3.2.1. Individual value bar graph representing a comparison of total circulating endothelial cells (CECs) (panel A), Annexin V^- CECs (panel B) and Annexin V^+ CECs (panel C) in healthy controls (n=25), stable native CAD patients (n=80), and heart transplant recipients with CAV (n=30). Data points show the individual natural logarithm (ln) transformed values. Means of ln transformed values are shown by the horizontal lines.

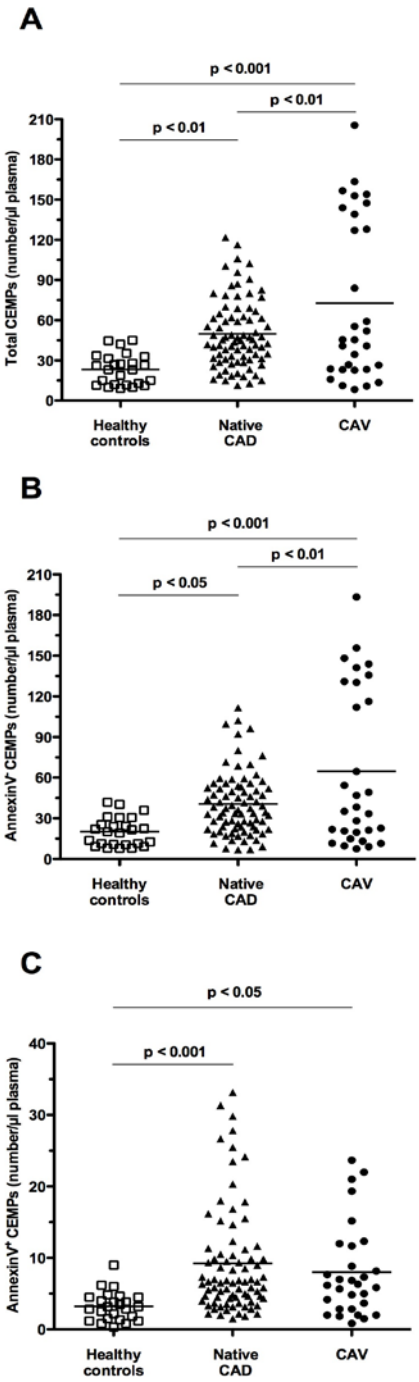

Figure 3.2.2. Individual value bar graph illustrating a comparison of total circulating endothelial microparticles (CEMPs) (panel A), Annexin V^- CEMPs (panel B) and Annexin V^+ CEMPs (panel C) in healthy controls (n=25), stable native CAD patients (n=80), and heart transplant recipients with CAV (n=30). Data points show the individual values. Means are shown by the horizontal lines.

3.2.4.4 Platelet microparticles are strikingly lower in patients with native CAD

Circulating platelet ($CD61^+$) microparticles (CPMPs), Annexin V negative $CD61^+$ CPMPs, and Annexin V positive $CD61^+$ CPMPs were significantly lower in patients with native CAD compared to healthy controls and transplant recipients with CAV as illustrated in Figure 3.2.3A, Figure 3.2.3B, and Figure 3.2.3C, respectively. The median value of total $CD61^+$ CPMPs in patients with native CAD was 69.4% ($p<0.001$) and 71.6% ($p<0.001$) lower compared to healthy controls and transplant recipients with CAV, respectively. This decline was observed both for Annexin V negative $CD61^+$ CPMPs (Figure 3.2.3B) and Annexin V positive $CD61^+$ CPMPs (Figure 3.2.3C). No difference in any of these parameters was observed between healthy controls and transplant recipients with CAV.

To confirm these pronounced differences, we additionally quantified the number of $CD42a^+CD31^+$ CPMPs. The median value of $CD42a^+CD31^+$ CPMPs in patients with native CAD was decreased by 95.9% ($p<0.001$) and by 89.2% ($p<0.001$) compared to healthy controls and heart transplant recipients with CAV, respectively (Figure 3.2.4A). Similarly to the decline of total $CD42a^+CD31^+$ CPMPs, Annexin V negative $CD42a^+CD31^+$ CPMPs (Figure 3.2.4B) and Annexin V positive $CD42a^+CD31^+$ CPMPs (Figure 3.2.4C) were greatly reduced in patients with native CAD compared to healthy controls and patients with CAV.

Since all patients with native CAD and only one patient with CAV were taking antiplatelet drugs, this suggested that the observed difference in CPMPs is due to this class of medication. The concentration of $CD61^+$ CPMPs (60.8/µl) and $CD42a^+CD31^+$ CPMPs (138/µl) in the CAV patient taking acetylsalicylic acid was in the order of magnitude of patients with native CAD (Figure 3.2.3A and 3.2.3B). To further confirm the effect of acetylsalicylic acid on CPMPs, an additional quantification was performed in 11 heart transplant recipients taking this drug. The median value and distribution (not shown) of $CD61^+$ CPMPs (53.0/µl) and of $CD42a^+CD31^+$ CPMPs (71.2/µl) in these heart transplant recipients was similar compared to patients with native CAD (Figure 3.2.3A, Figure 3.2.4A). In addition, the concentration of $CD61^+$ CPMPs (65.7/µl and 74.3/µl) and of $CD42a^+CD31^+$ CPMPs (52.3/µl and 56.2/µl) in two heart transplant recipients taking clopidogrel were close to the median values in patients with native CAD. All in all, very low concentrations of CPMPs in patients with native CAD very likely reflect intake of antiplatelet drugs.

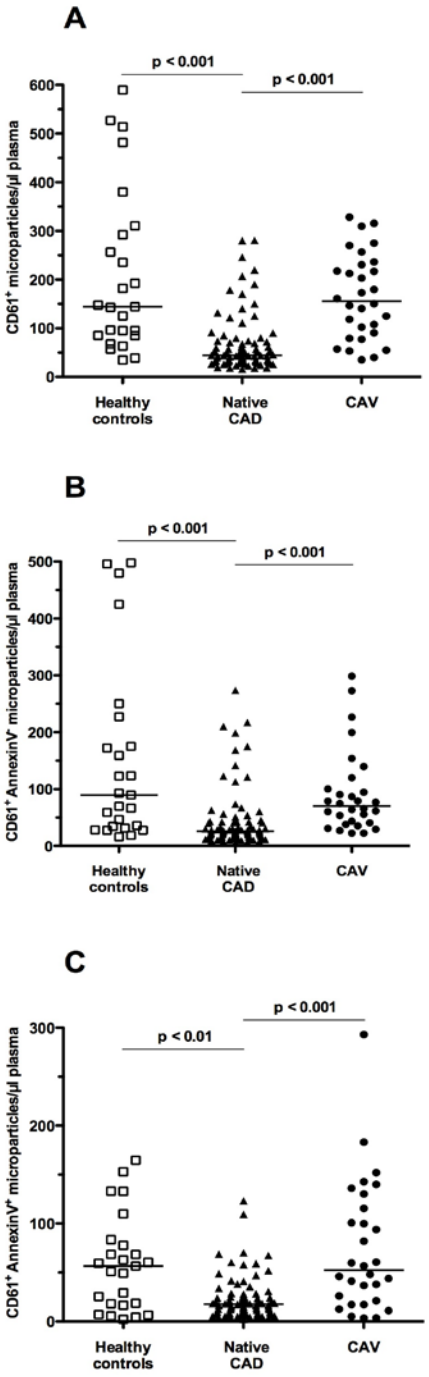

Figure 3.2.3. Individual value bar graph showing a comparison of $CD61^+$ platelet microparticles (panel A), $CD61^+$ Annexin V^- platelet microparticles (panel B) and $CD61^+$ Annexin V^+ platelet microparticles (panel C) in healthy controls (n=25), stable native CAD patients (n=80), and heart transplant recipients with CAV (n=30). Data points show the individual values. Medians are shown by the horizontal lines.

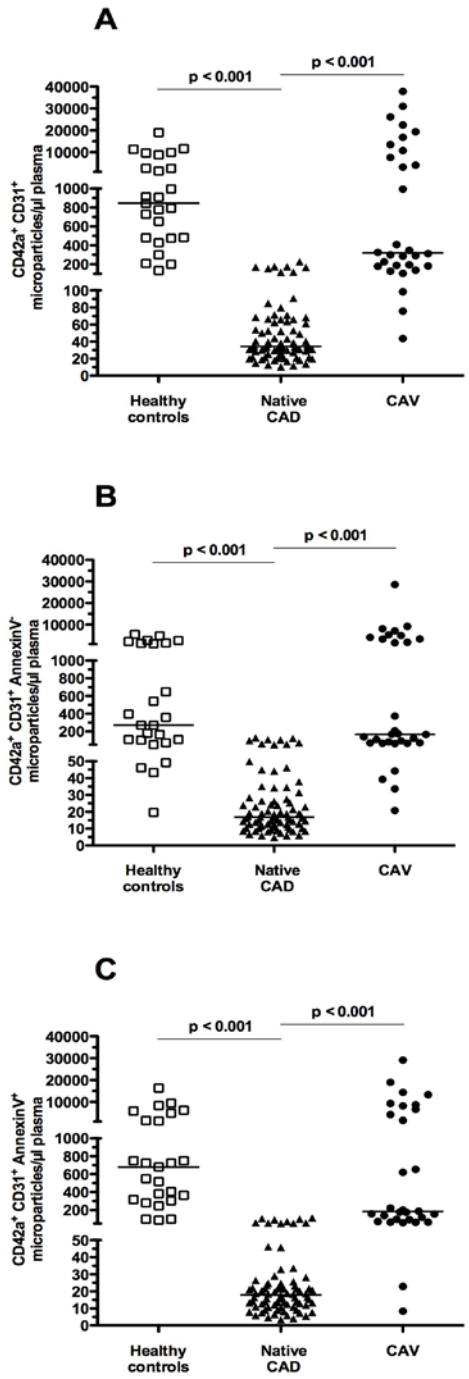

Figure 3.2.4. Individual value bar graph demonstrating a comparison of $CD42a^+$ $CD31^+$ platelet microparticles (panel A), $CD42a^+$ $CD31^+$ Annexin V^- platelet microparticles (panel B) and $CD42a^+$ $CD31^+$ Annexin V^+ platelet microparticles (panel C) in healthy controls (n=25), stable native CAD patients (n=80), and heart transplant recipients with CAV (n=30). Data points show the individual values. Medians are shown by the horizontal lines.

3.2.4.5 Evaluation of the relation of CECs, CEMPs, and CPMPs with clinical and laboratory parameters in patients with native CAD and transplant recipients with CAV

The relation between age, diabetes status, sex, creatinine levels, body mass index, and C-reactive protein levels on the one hand, and end-point parameters on the other hand was evaluated in patients with native CAD. The median value of total $CD61^+$ CPMPs was significantly higher ($p<0.05$) in women (61.7/µl) compared to men (43.3/µl). The median values of total $CD42a^+CD31^+$ CPMPs (43.8/µl versus 32.3/µl), Annexin V negative $CD42a^+CD31^+$ CPMPs (22.8/µl versus 15.0/µl), and Annexin V positive $CD42a^+CD31^+$ CPMPs (22.3/µl versus 15.8/µl) were significantly higher ($p<0.05$) in women compared to men. $CD61^+$ Annexin V^- CPMPs showed a weak positive correlation ($r=0.280$; $p=0.0118$) with body mass index in patients with native CAD. Total $CD42a^+CD31^+$ CPMPs and Annexin V^+ $CD42a^+CD31^+$ CPMPs weakly positively correlated with C-reactive protein levels ($r=0.260$; $p=0.0199$ and $r=0.360$; $p=0.0010$, respectively). Other comparisons did not show statistically significant differences in patients with native CAD.
In transplant recipients with CAV, a positive correlation was observed between creatinine levels and total CECs ($r=0.421$; $p=0.0205$), Annexin V negative CECs ($r=0.381$; $p=0.0377$), and Annexin V positive CEC ($r=0.466$; $p=0.0095$). In contrast, creatinine levels correlated negatively with total CEMPs ($r=-0.390$; $p=0.0331$) and Annexin V negative CEMPs ($r=-0.385$; $p=0.0358$). No other relationship was statistically significant.

3.2.5 DISCUSSION

The salient findings of the current study are that 1) patients with CAV display a distinct pattern of endothelial injury compared to patients with native CAD characterized by a more prominent increase of total CEMPs but a similar elevation of total CECs compared to healthy controls; 2) the selective increase of Annexin V negative CEMPs and the absence of a difference in Annexin V positive CECs strongly suggest increased endothelial cell activation but not endothelial cell apoptosis in CAV positive patients compared to stable CAD patients and 3) CPMPs are not elevated in transplant recipients with CAV compared to healthy controls and are markedly lower in patients with native CAD most probably as a result of generalized use of antiplatelet drugs.
Since CEMPs were higher in transplant recipients with CAV than in patients with native CAD whereas CECs were similar, the results of the current study are consistent with increased endothelial cell activation in the former. The endothelium regulates vascular tone, inflammation, smooth muscle cell proliferation, and thrombosis[23]. Endothelial dysfunction is considered to be pivotal in the pathogenesis of native CAD and CAV[2, 3]. It is characterized by loss of endothelium-dependent vasorelaxation, deprivation of anticoagulant, anti-inflammatory and antithrombotic properties, absence of antiproliferative effects on smooth muscle cells, and increased endothelial permeability. Endothelial dysfunction is paralleled by a switch from a quiescent to an activated phenotype characterized by expression of adhesion molecules, cytokines, chemokines, and

expression of major histocompatibility class (MHC II) molecules[3, 24]. Both native CAD and CAV are considered to be immune diseases[3, 15, 24]. However, the main antigens driving atherosclerosis are likely modified low density lipoproteins[25] whereas the principal antigen in CAV are nonself major histocompatibility complex molecules, especially HLA-DR, that are most abundantly expressed on luminal endothelial cells[26, 27]. Activated T cells in the subendothelium of allografts release cytokines including interferon-γ that promote endothelial cell activation[27]. Consistent with the different nature of the antigen, the inflammatory infiltrate is localized in the subendothelium in CAV whereas T cells and macrophages are largely localized in the shoulder regions of atheromata[27]. The higher degree of endothelial cell activation in CAV as evidenced by increased CEMPs may reflect both the localization and the nature of the alloantigen eliciting an adaptive immune response.

To further investigate a distinct pattern of endothelial injury in patients with native CAD and in transplant recipients with CAV, Annexin V negative CEMPs and Annexin V positive CEMPs were quantified. It has been proposed that not only cells undergoing apoptosis but also activated cells are characterized by a loss of asymmetry of normal cell membrane phospholipids, resulting in an increase of phosphatidylserine on the outer leaflet of the bilipid cell membrane[28]. At least theoretically, endothelial cell activation may also give rise to Annexin V positive CEMPs. However, the large majority of CEMPs in the current study as well as in previous studies are Annexin V negative[29]. This suggests that the *in vivo* vesiculation process causing microparticle formation in activated endothelial cells can occur independently of membrane asymmetry loss[30]. In agreement with this, it has been shown that Annexin V binding sites on endothelial cells occur only in apoptosis[31]. Taken together, the selective increase of Annexin V negative CEMPs in transplant recipients with CAV compared to native CAD patients strongly suggests a difference in endothelial cell activation but no difference in endothelial cell apoptosis. This is consistent with the observation that no significant difference in Annexin V positive CECs was observed. The absence of increased endothelial cell apoptosis in CAV may be surprising in light of the alloimmune response against endothelial cells. However, there is little evidence for cytolysis in CAV lesions. Cytotoxicity is typically mediated by CD8 expressing T cells but apparently, activated T cells in CAV are cytokine-producing $CD4^+$ cells[27].

Sloughing off endothelial cells may not only occur by apoptosis but also as a result of biomechanical injury, defective adhesive properties of endothelial cells, or protease- or cytokine mediated detachment[14]. The data on Annexin V negative CECs indicate no difference in non-apoptotic endothelial cell detachment between patients with CAV and with native CAD. Taken together, irreversible endothelial damage is similar in both conditions.

The interaction of platelets with endothelial cells and leukocytes is considered to be important for the initiation and progression of atherogenesis[32]. Quiescent endothelial cells counteract platelet adhesion via production of nitric oxide and of prostacyclin and via the ecto-ADPase/CD39/NTPDase pathway[33]. These anti-adhesive properties are lost in activated endothelium. Activated platelets may promote atherosclerosis or fibromuscular intimal hyperplasia via release of mitogenic and inflammatory mediators[34]. Endothelial-bound platelets are highly

effective at recruiting leukocytes from flowing blood[35]. CPMPs are the most abundant microparticle subtype[36, 37]. A remarkable finding of the current study is that CPMPs were not increased at all in heart transplant recipients with CAV compared to healthy controls. Furthermore, levels of $CD61^+$ CPMPs were markedly lower and levels of $CD42a^+CD31^+$ CPMPs were dramatically lower in patients with stable native CAD compared to CAV-positive patients and healthy controls. The much more pronounced decline of $CD42a^+CD31^+$ CPMPs might be in part related to shedding of the von Willebrand Factor receptor complex (glycoprotein Ib-V-IX) via ADAM17 induced by acetylsalicylic acid[38]. However, it is rather unlikely that this particular mechanism occurs at the low dosages of acetylsalicylic acid taken by these patients. This is corroborated by the observation that similar values of $CD42a^+CD31^+$ CPMPs were observed in two heart transplant recipients taking clopidogrel. Taken together, the data on CPMPs do not support a plausible role of CPMPs in the pathogenesis of these two types of arteriosclerosis. The data provide converging evidence that generalized use of antiplatelet drugs in patients with native CAD underlies low CPMPs.

In conclusion, the selective increase of Annexin V negative CEMPs and the absence of an increase of Annexin V positive CECs in transplant recipients with CAV compared to native CAD is indicative of increased endothelial cell activation without increased endothelial cell apoptosis. This likely reflects the nature and the localization of the eliciting alloantigen in CAV and the fact activated T cells in CAV are cytokine-producing $CD4^+$ cells. We also describe for the first time that CPMPs are strikingly lower in patients with native CAD most likely as a result of generalized use of antiplatelet drugs.

3.2.6 REFERENCES

1. Lu, W.H., Palatnik, K., Fishbein, G.A., Lai, C., Levi, D.S., Perens, G., Alejos, J., Kobashigawa, J. ,Fishbein, M.C. Diverse morphologic manifestations of cardiac allograft vasculopathy: a pathologic study of 64 allograft hearts. *J Heart Lung Transplant* **30**, 1044-1050 (2011).
2. Ross, R. Atherosclerosis--an inflammatory disease. *N Engl J Med* **340**, 115-126. (1999).
3. Schmauss, D. ,Weis, M. Cardiac allograft vasculopathy: recent developments. *Circulation* **117**, 2131-2141 (2008).
4. Ross, R. ,Glomset, J.A. Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. *Science* **180**, 1332-1339 (1973).
5. Ross, R. ,Glomset, J.A. The pathogenesis of atherosclerosis (first of two parts). *N Engl J Med* **295**, 369-377 (1976).
6. Ross, R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* **362**, 801-809. (1993).
7. Burger, D. ,Touyz, R.M. Cellular biomarkers of endothelial health: microparticles, endothelial progenitor cells, and circulating endothelial cells. *J Am Soc Hypertens* **6**, 85-99 (2012).
8. Mause, S.F. ,Weber, C. Microparticles: protagonists of a novel communication network for intercellular information exchange. *Circ Res* **107**, 1047-1057 (2010).
9. Amabile, N., Guerin, A.P., Leroyer, A., Mallat, Z., Nguyen, C., Boddaert, J., London, G.M., Tedgui, A. ,Boulanger, C.M. Circulating endothelial microparticles are associated with vascular dysfunction in patients with end-stage renal failure. *J Am Soc Nephrol* **16**, 3381-3388 (2005).
10. Esposito, K., Ciotola, M., Schisano, B., Gualdiero, R., Sardelli, L., Misso, L., Giannetti, G. ,Giugliano, D. Endothelial microparticles correlate with endothelial dysfunction in obese women. *J Clin Endocrinol Metab* **91**, 3676-3679 (2006).
11. Feng, B., Chen, Y., Luo, Y., Chen, M., Li, X. ,Ni, Y. Circulating level of microparticles and their correlation with arterial elasticity and endothelium-dependent dilation in patients with type 2 diabetes mellitus. *Atherosclerosis* **208**, 264-269 (2010).
12. Werner, N., Wassmann, S., Ahlers, P., Kosiol, S. ,Nickenig, G. Circulating CD31+/annexin V+ apoptotic microparticles correlate with coronary endothelial function in patients with coronary artery disease. *Arterioscler Thromb Vasc Biol* **26**, 112-116 (2006).
13. Boos, C.J., Lip, G.Y. ,Blann, A.D. Circulating endothelial cells in cardiovascular disease. *J Am Coll Cardiol* **48**, 1538-1547 (2006).
14. Blann, A.D., Woywodt, A., Bertolini, F., Bull, T.M., Buyon, J.P., Clancy, R.M., Haubitz, M., Hebbel, R.P., Lip, G.Y., Mancuso, P., Sampol, J., Solovey, A. ,Dignat-George, F. Circulating endothelial cells. Biomarker of vascular disease. *Thromb Haemost* **93**, 228-235 (2005).

15. Rogers, N.J. ,Lechler, R.I. Allorecognition. *Am J Transplant* **1**, 97-102 (2001).
16. Singh, N., Van Craeyveld, E., Tjwa, M., Ciarka, A., Emmerechts, J., Droogne, W., Gordts, S.C., Carlier, V., Jacobs, F., Fieuws, S., Vanhaecke, J., Van Cleemput, J. ,De Geest, B. Circulating apoptotic endothelial cells and apoptotic endothelial microparticles independently predict the presence of cardiac allograft vasculopathy. *J Am Coll Cardiol* **60**, 324-331 (2012).
17. Mehra, M.R., Crespo-Leiro, M.G., Dipchand, A., Ensminger, S.M., Hiemann, N.E., Kobashigawa, J.A., Madsen, J., Parameshwar, J., Starling, R.C. ,Uber, P.A. International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for cardiac allograft vasculopathy-2010. *J Heart Lung Transplant* **29**, 717-727 (2010).
18. Duda, D.G., Cohen, K.S., Scadden, D.T. ,Jain, R.K. A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. *Nat Protoc* **2**, 805-810 (2007).
19. Pereira, J., Alfaro, G., Goycoolea, M., Quiroga, T., Ocqueteau, M., Massardo, L., Perez, C., Saez, C., Panes, O., Matus, V. ,Mezzano, D. Circulating platelet-derived microparticles in systemic lupus erythematosus. Association with increased thrombin generation and procoagulant state. *Thromb Haemost* **95**, 94-99 (2006).
20. Craft, J.A. ,Marsh, N.A. Increased generation of platelet-derived microparticles following percutaneous transluminal coronary angioplasty. *Blood Coagul Fibrinolysis* **14**, 719-728 (2003).
21. Brogan, P.A., Shah, V., Brachet, C., Harnden, A., Mant, D., Klein, N. ,Dillon, M.J. Endothelial and platelet microparticles in vasculitis of the young. *Arthritis Rheum* **50**, 927-936 (2004).
22. Kang, P., Shen, B., Yang, J. ,Pei, F. Circulating platelet-derived microparticles and endothelium-derived microparticles may be a potential cause of microthrombosis in patients with osteonecrosis of the femoral head. *Thromb Res* **123**, 367-373 (2008).
23. Behrendt, D. ,Ganz, P. Endothelial function. From vascular biology to clinical applications. *Am J Cardiol* **90**, 40L-48L (2002).
24. Hansson, G.K. Atherosclerosis--an immune disease: The Anitschkov Lecture 2007. *Atherosclerosis* **202**, 2-10 (2009).
25. Libby, P., Lichtman, A.H. ,Hansson, G.K. Immune effector mechanisms implicated in atherosclerosis: from mice to humans. *Immunity* **38**, 1092-1104 (2013).
26. Kaczmarek, I., Deutsch, M.A., Rohrer, M.E., Beiras-Fernandez, A., Groetzner, J., Daebritz, S., Schmoeckel, M., Spannagl, M., Meiser, B. ,Reichart, B. HLA-DR matching improves survival after heart transplantation: is it time to change allocation policies? *J Heart Lung Transplant* **25**, 1057-1062 (2006).
27. Pober, J.S., Jane-Wit, D., Qin, L. ,Tellides, G. Interacting Mechanisms in the Pathogenesis of Cardiac Allograft Vasculopathy. *Arterioscler Thromb Vasc Biol* **34**, 1609-1614 (2014).

28. Zwaal, R.F. ,Schroit, A.J. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood* **89**, 1121-1132 (1997).
29. Dignat-George, F. ,Boulanger, C.M. The many faces of endothelial microparticles. *Arterioscler Thromb Vasc Biol* **31**, 27-33 (2011).
30. Shet, A.S., Aras, O., Gupta, K., Hass, M.J., Rausch, D.J., Saba, N., Koopmeiners, L., Key, N.S. ,Hebbel, R.P. Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes. *Blood* **102**, 2678-2683 (2003).
31. Jimenez, J.J., Jy, W., Mauro, L.M., Soderland, C., Horstman, L.L. ,Ahn, Y.S. Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. *Thromb Res* **109**, 175-180 (2003).
32. Borissoff, J.I., Spronk, H.M. ,ten Cate, H. The hemostatic system as a modulator of atherosclerosis. *N Engl J Med* **364**, 1746-1760 (2011).
33. Jin, R.C., Voetsch, B. ,Loscalzo, J. Endogenous mechanisms of inhibition of platelet function. *Microcirculation* **12**, 247-258 (2005).
34. Langer, H.F. ,Gawaz, M. Platelet-vessel wall interactions in atherosclerotic disease. *Thromb Haemost* **99**, 480-486 (2008).
35. Kaplan, Z.S. ,Jackson, S.P. The role of platelets in atherothrombosis. *Hematology Am Soc Hematol Educ Program* **2011**, 51-61 (2011).
36. Orozco, A.F. ,Lewis, D.E. Flow cytometric analysis of circulating microparticles in plasma. *Cytometry A* **77**, 502-514 (2010).
37. Horstman, L.L. ,Ahn, Y.S. Platelet microparticles: a wide-angle perspective. *Crit Rev Oncol Hematol* **30**, 111-142 (1999).
38. Aktas, B., Pozgajova, M., Bergmeier, W., Sunnarborg, S., Offermanns, S., Lee, D., Wagner, D.D. ,Nieswandt, B. Aspirin induces platelet receptor shedding via ADAM17 (TACE). *J Biol Chem* **280**, 39716-39722 (2005).

3.3 IMPAIRED CHOLESTEROL EFFLUX CAPACITY AND VASCULOPROTECTIVE FUNCTION OF HDL IN HEART TRANSPLANT RECIPIENTS*

3.3.1 ABSTRACT

Background: High-density lipoprotein (HDL) metabolism is significantly altered in heart transplant recipients. We hypothesized that HDL function may be impaired in these patients.

Methods: Fifty-two patients undergoing coronary angiography between 5 and 15 years after heart transplantation were recruited in this cross-sectional study. Cholesterol efflux capacity of apolipoprotein B-depleted plasma was analysed using a validated assay. The vasculoprotective function of HDL was studied by means of an endothelial progenitor cell migration assay.

Results: HDL cholesterol levels were similar in heart transplant patients compared to healthy controls. However, normalized cholesterol efflux and vasculoprotective function were reduced by 24.1% ($p<0.001$) and by 27.0% ($p<0.01$), respectively, in heart transplant recipients compared to healthy controls. HDL function was similar in patients with and without cardiac allograft vasculopathy (CAV) and was not related to C-reactive protein (CRP) levels. An interaction effect ($p=0.0584$) was observed between etiology of heart failure before transplantation and steroid use as factors of HDL cholesterol levels. Lower HDL cholesterol levels occurred in patients with prior ischemic cardiomyopathy not taking steroids. However, HDL function was independent of the etiology of heart failure before transplantation and steroid use. The percentage of patients with a CRP level greater than or equal to 6 mg/l was 3.92-fold ($p<0.01$) higher in patients with CAV than in patients without CAV.

Conclusions: HDL function is impaired in heart transplant recipients but is unrelated to CAV-status. The proportion of patients with a CRP level greater than or equal to 6 mg/l is prominently higher in CAV-positive patients.

3.3.2 INTRODUCTION

The long-term success of heart transplantation is limited by cardiac allograft vasculopathy (CAV), which is characterized by the coexistence of diffuse fibromuscular intimal hyperplasia and focal atherosclerosis[1, 2]. The pathogenesis of CAV predominantly involves alloimmunity but is modified by non-immunological factors including metabolic abnormalities. The prevalence and the incidence of CAV have been reported to be increased in heart transplant recipients with decreased high density lipoprotein (HDL) cholesterol levels[3-6]. The association between HDL cholesterol and CAV

* This chapter has been published as: **Singh, N.**, Jacobs, F., Rader, D., Vanhaecke, J., Van Cleemput, J., De Geest, B. Impaired cholesterol efflux capacity and vasculoprotective function of HDL in heart transplant recipients. *Journal of Heart and Lung Transplantation* 2014; 33 (5), 499-506.

may reflect causation but might also be due to residual confounding. One such confounding factor is insulin resistance, which is considered to play a role in the pathogenesis of CAV. A triglyceride/HDL cholesterol ratio of greater than 3 has been recognized as a marker of insulin resistance in overweight subjects[7] and constituted a risk factor for CAV and major adverse cardiac events in heart transplant recipients[8, 9].

Remodelling of HDL in heart transplant recipients is significantly affected by a lower activity of cholesterol ester transfer protein, phospholipid transfer protein, and hepatic lipase[10, 11]. Consequently, these patients are characterized by an increased proportion of large HDL particles and reduced pre-ß1-HDL in the presence of normal or even elevated HDL cholesterol levels[10, 11]. These alterations may be partially explained by corticosteroid use[12] but may also be potentiated by statin intake[13]. The modified HDL metabolism and associated compositional changes of HDL particles may lead to an impaired function of these lipoproteins. Reduced HDL function may also occur as a result of ongoing inflammation[14].

The current cross-sectional study evaluated the hypothesis that HDL is dysfunctional in heart transplant recipients. To assess cholesterol efflux capacity of HDL, we used a validated assay that was designed to integrate the efflux pathways thought to be operative *in vivo*[15]. Cholesterol efflux capacity analyzed by this assay was a stronger predictor of prevalent atherosclerotic burden than HDL cholesterol or apolipoprotein (apo) A-I[15]. Since cumulative endothelial injury induced by both alloimmune responses and non-alloimmune insults is thought to be central in the pathogenesis of CAV[1, 16], we also assessed the vasculoprotective function of HDL by use of an endothelial progenitor cell (EPC) migration assay[17, 18]. Heart transplant recipients were compared with a healthy control reference group. Furthermore, we investigated the hypothesis that HDL function would be worse in CAV-positive patients than in CAV-negative patients.

3.3.3 METHODS

3.3.3.1 Study design

Fifty-two clinically stable patients undergoing coronary angiography between 5 and 15 years after heart transplantation were recruited in this cross-sectional study. The clinical characteristics of CAV-negative and CAV-positive patients have been described in a previous report[19]. Heart transplant patients with prior congenital heart disease and re-transplanted patients were excluded. The study was approved by the Ethics Committee of the University Hospital Gasthuisberg and written informed consent was obtained from all participating subjects. The reference control group comprised 25 healthy subjects.

CAV was graded according to the International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for CAV-2010[20]. Patients with no detectable angiographic lesions (CAV_0) constitute the CAV-negative group, whereas CAV-positive group consisted of patients with CAV_1, CAV_2, and CAV_3.

3.3.3.2 Analysis of cholesterol efflux capacity

Apo B-depleted plasma was obtained after removal of apo B containing lipoproteins with polyethylene glycol (molecular weight 8000) (Sigma-Aldrich, Saint Louis, MO, USA) as described[21]. Cholesterol efflux capacity was analyzed essentially as described before by Khera *et al.*[22]. J774 cells, derived from a murine macrophage cell line, were plated and radiolabeled with 2 µCi of ^{3}H-cholesterol (Perkin Elmer, Waltham, Massachussetts, USA) per ml and incubated for 16 hours at 37°C. Next, 0.3 mM 8-(4-chlorophenylthio)-cyclic AMP (Sigma-Aldrich) was added for 6 hours to up-regulate ABCA1. Subsequently, efflux media containing 2.8% apo B-depleted plasma were added to the cells. Different steps were performed in the presence of the acyl–coenzyme A:cholesterol acyltransferase inhibitor CP113,818 (2 µg per ml). Each sample was run in duplicate. Radioactive cholesterol was quantified by liquid scintillation counting. The quantity of radioactive cholesterol incorporated into cellular lipids was calculated by means of extraction with 0.2N NaOH/1% sodium dodecyl sulfate for 30 minutes. Percent efflux was calculated as follows: [(microcuries of ^{3}H -cholesterol in medium containing 2.8% apo B–depleted plasma − microcuries of ^{3}H -cholesterol in plasma-free medium) / (microcuries of ^{3}H -cholesterol in cellular extract of wells not exposed to efflux phase)] x 100. Values of percent efflux of individuals were normalized to the value of percent efflux of a plasma pool of 14 healthy individuals that was included in each plate to correct for interassay variation.

3.3.3.3 Evaluation of the vasculoprotective function of HDL

The vasculoprotective function of HDL was studied by means of an EPC migration assay as described[17, 18]. Male C57BL/6 mice were used as a homogeneous source of EPCs. Apo B-depleted plasma was filtered through a 50K membrane (Amicon Ultra; Merck Millipore, Billerica, MA, USA). HDL retained on top of the filter was added in the lower chamber at 20% of the original plasma concentration. EPCs were allowed to migrate for 5 hours at 37 °C. For quantification, cell nuclei were stained with 4',6-diamidine-2-phenylidole dihydrochloride (DAPI) (Vector Laboratories, Burlingame, CA, USA) and EPCs migrated into the lower chamber were counted manually in 7 randomly selected microscopy fields. All experimental conditions were performed in duplicate.

3.3.3.4 Quantification of human apo A-I in plasma by sandwich enzyme-linked immunosorbent assay (ELISA)

Human apo A-I levels in plasma were determined by sandwich ELISA[23]. The sensitivity of this assay is 30 µg/ml.

3.3.3.5 Statistical analysis

Fisher's exact test was used to compare categorical data between two groups using Instat 3 (Graphpad software, San Diego, CA, USA). Continuous variables were summarized by means, standard error of the mean, and sample size, and were compared between two groups by an unpaired t-test. If indicated, a transformation (natural logarithm) was applied. Continuous parameters between 3 groups were compared by analysis of variance followed by Tukey's multiple comparison post-test. Two-way ANOVA test for interaction was performed using Prism4 (GraphPad Software, San Diego, CA, USA). A p-value of less than 0.05 was considered statistically significant.

3.3.4 RESULTS

3.3.4.1 Characteristics of heart transplant recipients included in the cross-sectional study

Clinical characteristics, laboratory parameters, and immunosuppressive and hypolipidemic therapy of heart transplant recipients (n=52) are summarized in Table 3.3.1. Patients were also categorized according to heart failure etiology before transplantation. Patients with prior ischemic cardiomyopathy (n=23) were older and had higher serum creatinine levels than patients with prior non-ischemic cardiomyopathy (n=29). Other parameters did not differ between both groups.

3.3.4.2 Comparison of lipoprotein parameters in healthy controls and heart transplant recipients

Lipoprotein parameters in healthy controls (n=25) and heart transplant recipients (n=52) are shown in Table 3.3.2. Serum cholesterol and low density lipoprotein (LDL) cholesterol levels were 18.7% ($p<0.001$) and 27.9% ($p<0.0001$) lower in heart transplant recipients than in healthy controls, reflecting generalized statin use in patients (98.1%). HDL cholesterol levels and the proportion of individuals with a triglyceride/HDL cholesterol ratio greater than or equal to 3 were not significantly different between both groups. Apo A-I levels were 11.5% ($p<0.05$) lower in heart transplant recipients than in controls. Since the proportion of male subjects was higher in the healthy control group, a separate analysis was performed in male subjects. The difference of apo A-I levels was 11.7% ($p=0.0687$) when male healthy controls (n=12) and male transplant recipients (n=42) were compared.

3.3.4.3 Comparison of lipoprotein parameters and C-reactive protein levels between CAV-negative and CAV-positive heart transplant recipients

A comparison of lipoprotein parameters and C-reactive protein (CRP) level between CAV-negative and CAV-positive heart transplant recipients is illustrated in Table 3.3.3. LDL cholesterol levels were 15.9% ($p<0.05$) lower in patients with CAV than in patients without CAV, reflecting a policy to switch to more potent statins after the diagnosis of CAV. The proportion of patients with a triglyceride/HDL cholesterol ratio greater than or equal to 3 was not significantly different between CAV-negative and CAV-positive patients. The median CRP level was 2.24-fold ($p=0.082$) higher in patients with CAV than in patients without CAV.

Table 3.3.1. Clinical characteristics, laboratory parameters, and immunosuppressive and hypolipidemic therapy of all heart transplant recipients and of patients dichotomized according to heart failure etiology.

	All heart transplant recipients (n=52)	Prior ischemic cardiomyopathy (n=23)	Non-ischemic cardiomyopathy (n=29)	P value
Age at inclusion in the study (years)	61.3 ± 1.9	64.9 ± 1.5	58.4 ± 3.1	0.0489
Sex (male/female)	80.8%/19.2%	95.7%/4.3%	69.0%/31.0%	0.0300
Donor age (years)	37.9 ± 1.9	37.4 ± 2.8	38.2 ± 2.6	0.848
Time after heart transplantation (years)	10.2 ± 0.5	10.4 ± 0.7	10.1 ± 0.7	0.813
Sex mismatch graft (%)	17.3%	13.0%	20.7%	0.714
Acute rejection episodes (Grade 3A or > 3A)	11.5%	13.0%	10.3%	1.00
Current smoker (%)	5.77%	8.70%	3.45%	0.578
Hypertension (%)	96.2%	95.7%	96.6%	1.00
Diabetes (%)	28.9%	39.1%	20.7%	0.218
Body mass index (kg/m^2)	26.0 ± 0.4	26.6 ± 0.7	25.5 ± 0.6	0.215
Platelet count (10^9/L)	224 ± 10	219 ± 12	227 ± 15	0.672
Leukocyte count (10^9/L)	6.84 ± 0.20	6.90 ± 0.31	6.80 ± 0.25	0.789
Monocyte count (10^9/L)	0.322 ± 0.030	0.315 ± 0.043	0.328 ± 0.043	0.843
Lymphocyte count (10^9/L)	1.27 ± 0.09	1.37 ± 0.15	1.18 ± 0.11	0.294
Neutrophil count (10^9/L)	3.50 ± 0.25	3.24 ± 0.32	3.71 ± 0.37	0.343
Creatinine (mg/dl)	1.48 ± 0.07	1.65 ± 0.11	1.35 ± 0.07	0.0212
Statins (%)	98.1%	100%	96.6%	1.00
Cyclosporine (%)	26.9%	30.4%	24.1%	0.755
Tacrolimus (%)	67.3%	60.9%	72.4%	0.553
Everolimus (%)	13.5%	17.4%	10.3%	0.686
Azathioprine (%)	11.5%	21.7%	3.45%	0.0762
Mycophenolate mofetil (%)	71.2%	73.9%	69.0%	0.765
Steroid (%)	44.2%	34.8%	51.7%	0.269

Data of continuous variables represent means ± SEM. P values represent the comparison of patients transplanted for ischemic and for non-ischemic cardiomypathy.

Table 3.3.2. Comparison of lipoprotein parameters in healthy controls and heart transplant recipients.

	Healthy controls (n=25)	Transplant recipients (n=52)	P value
Age at inclusion in the study (years)	43.2 ± 2.0	61.3 ± 1.9	<0.0001
Sex (male/female)	12 (48.0%)/13 (52%)	42 (80.8%)/10 (19.2%)	0.0068
Cholesterol (mg/dl)	196 ± 8	161 ± 5	0.0002
Triglycerides (mg/dl)	104 ± 8	118 ± 8	0.192
HDL cholesterol (mg/dl)	59.5 ± 3.0	54.6 ± 2.3	0.206
LDL cholesterol (mg/dl)	115 ± 7	83.3 ± 3.4	<0.0001
Triglyceride/HDL cholesterol ratio	1.91 ± 0.21	2.36 ± 0.18	0.0994
Triglyceride/HDL cholesterol ratio ≥ 3	4 (16.0%)	13 (25.0%)	0.558
Apolipoprotein A-I (mg/dl)	137 ± 5	121 ± 4	0.0235

Data are expressed as means ± SEM for continuous variables.

Table 3.3.3. Comparison of lipoprotein parameters and CRP level between CAV-negative and CAV-positive heart transplant recipients.

	Patients without CAV (n=22)	Patients with CAV (n=30)	P value
Age at inclusion in the study (years)	56.3 ± 3.2	64.9 ± 2.1	0.0212
Sex (male/female)	18 (81.8%)/4 (18.2%)	24 (80.0%)/6 (20.0%)	1.00
Cholesterol (mg/dl)	168 ± 8	156 ± 6	0.211
Triglycerides (mg/dl)	108 ± 7	125 ± 12	0.244
HDL cholesterol (mg/dl)	55.0 ± 2.9	54.2 ± 3.4	0.487
LDL cholesterol (mg/dl)	91.7 ± 5.7	77.1 ± 4.0	0.0436
Triglyceride/HDL cholesterol ratio	2.08 ± 0.17	2.57 ± 0.28	0.144
Triglyceride/HDL cholesterol ratio ≥ 3	3 (13.6%)	9 (30.0%)	0.200
Apolipoprotein A-I (mg/dl)	128 ± 6	117 ± 5	0.176
CRP (mg/l)	2.99 (1.85-6.65)	6.71 (3.25-12.0)	0.0817

Data are expressed as means ± SEM for continuous variables or median (IQR).

3.3.4.4 Cholesterol efflux capacity and vasculoprotective function of HDL are impaired in heart transplant recipients but do not differ between CAV-negative and CAV-positive patients

Normalized cholesterol efflux was reduced by 24.1% ($p<0.001$) in heart transplant recipients compared to healthy controls (Figure 3.3.1A). This difference was 27.8% ($p<0.001$) when male patients (n=42) were compared with male healthy controls (n=12). No significant difference of normalized cholesterol efflux was observed between CAV-negative and CAV-positive patients (Figure 3.3.1B).

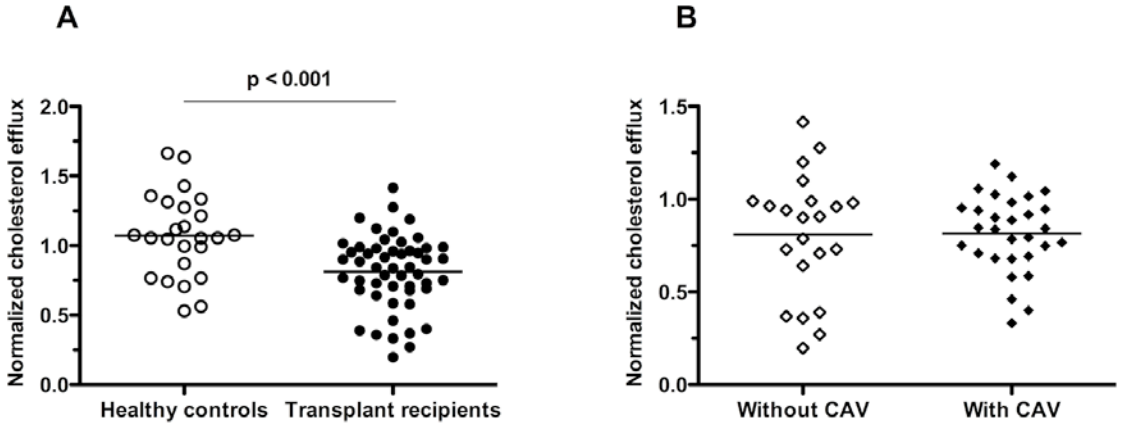

Figure 3.3.1. Individual value bar graph of normalized cholesterol efflux capacity values in healthy controls versus heart transplant recipients (panel A) and in CAV-negative versus CAV-positive patients (panel B). Data points show the individual values. Means are shown by the horizontal lines.

The vasculoprotective function of HDL was evaluated by EPC migration assay in modified Boyden chambers. Figure 3.3.2A shows a comparison of reference values in the absence of HDL and the number of migrated cells in the presence of HDL from healthy controls and transplant recipients. EPC migration was increased by 2.49-fold ($p<0.001$) and 1.96-fold ($p<0.001$) by the addition of HDL of healthy controls and heart transplant recipients, respectively, at 20% of the plasma concentration. EPC migration in the presence of HDL of healthy controls was 27.0% ($p<0.01$) higher than after addition of HDL of heart transplant recipients. When the analysis was restricted to male subjects, EPC migration following addition of HDL from healthy individuals was 39.9% ($p<0.01$) higher compared to HDL from heart transplant patients. EPC migration induced by HDL of patients with CAV was similar compared to EPC migration induced by HDL of patients without CAV (Figure 3.3.2B). The vasculoprotective function of HDL was not significantly correlated with cholesterol efflux capacity and HDL function was not related to CRP levels (data not shown). Taken together, generalized HDL dysfunction is observed in heart transplant recipients.

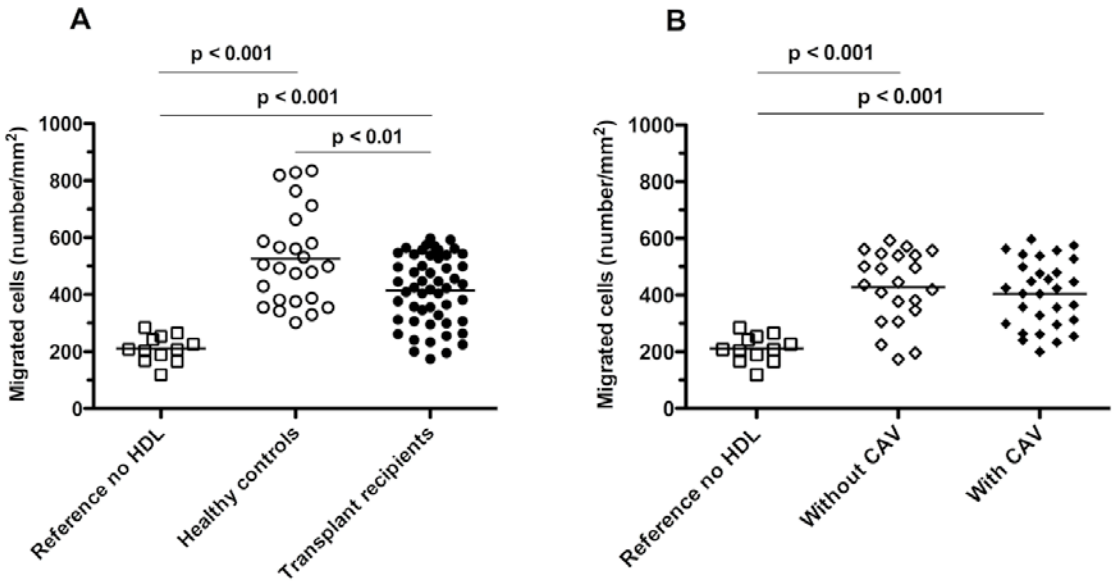

Figure 3.3.2. Individual value bar graph of vasculoprotective function of HDL evaluated by EPC migration assay in modified Boyden chambers. Panel A compares reference values in the absence of HDL and the number of migrated cells in the presence of HDL from healthy controls and heart transplant recipients. Panel B shows a comparison of reference values in the absence of HDL and the number of migrated cells in the presence of HDL from patients without CAV and patients with CAV. Data points show the individual values. Means are shown by the horizontal lines.

3.3.4.5 HDL cholesterol levels are markedly lower in patients with prior ischemic cardiomyopathy than in patients with prior non-ischemic cardiomyopathy but HDL function is similar in both groups

Since HDL is an independent risk marker for coronary artery disease, we investigated the hypothesis that HDL cholesterol levels and HDL function may differ between patients with prior ischemic and prior non-ischemic cardiomyopathy. Table 3.3.4 shows a comparison of lipoprotein parameters, HDL function, and CRP level between both groups. HDL cholesterol levels were 19.7% ($p<0.01$) lower in patients transplanted for ischemic cardiomyopathy than in patients transplanted for non-ischemic cardiomyopathy. This difference was 17.6% ($p<0.05$) when the analysis was restricted to male subjects. The percentage of patients with a triglyceride/HDL cholesterol ratio greater than or equal to 3 was 3.80-fold ($p<0.05$) higher in transplant recipients with prior ischemic cardiomyopathy than in patients with prior non-ischemic cardiomyopathy. Notwithstanding markedly lower HDL cholesterol levels in patients transplanted for ischemic cardiomyopathy, no difference in cholesterol efflux capacity or vasculoprotective function of HDL was observed.

Table 3.3.4. Comparison of lipoprotein parameters, HDL function, and CRP level between patients with ischemic and non-ischemic cardiomyopathy.

	Ischemic Cardiomyopathy (n=23)	Non-ischemic cardiomyopathy (n=29)	P value
Cholesterol (mg/dl)	152 ± 7	168 ± 7	0.352
Triglycerides (mg/dl)	128 ± 15	109 ± 7	0.102
HDL cholesterol (mg/dl)	48.0 ± 3.0	59.7 ± 3.1	0.0093
LDL cholesterol (mg/dl)	78.5 ± 4.3	87.0 ± 5.1	0.220
Triglyceride/HDL cholesterol ratio	2.87 ± 0.31	1.96 ± 0.16	0.0080
Triglyceride/HDL cholesterol ratio ≥ 3	9 (39.1%)	3 (10.3%)	0.0210
Apolipoprotein A-I (mg/dl)	115 ± 5	126 ± 6	0.173
CRP (mg/l)	9.3 ± 1.7	10.1 ± 3.4	0.435
CRP ≥ 6 mg/l	11 (47.8%)	8 (27.6%)	0.157
Normalized cholesterol efflux	0.802 ± 0.051	0.821 ± 0.053	0.793
Vasculoprotective function of HDL (migrated cells/mm^2)	430 ± 26	401 ± 22	0.392

Data are expressed as means ± SEM for continuous variables.

3.3.4.6 Steroid use is associated with markedly higher HDL cholesterol levels but does not affect HDL function

Since the effect of steroids on HDL metabolism may be an important modifier of HDL cholesterol levels, a comparison of lipoprotein parameters, HDL function, and CRP levels was performed between transplant recipients on steroids (n=23) and those not on steroids (n=29) (Table 3.3.5). HDL cholesterol levels were 16.5% ($p<0.06$) higher in patients on steroids compared to patients not on steroids. However, HDL function was similar in both groups. Interestingly, when separate analyses were performed in patients with prior ischemic cardiomyopathy and prior non-ischemic cardiomyopathy, an interaction effect ($p=0.0584$) was observed between etiology of heart failure before transplantation and steroid use as factors of HDL cholesterol levels. Decreased HDL cholesterol levels occurred in patients with prior ischemic cardiomyopathy not taking steroids. (Figure 3.3.3). However, this did not result in a different HDL function compared to the other 3 groups (data not shown).

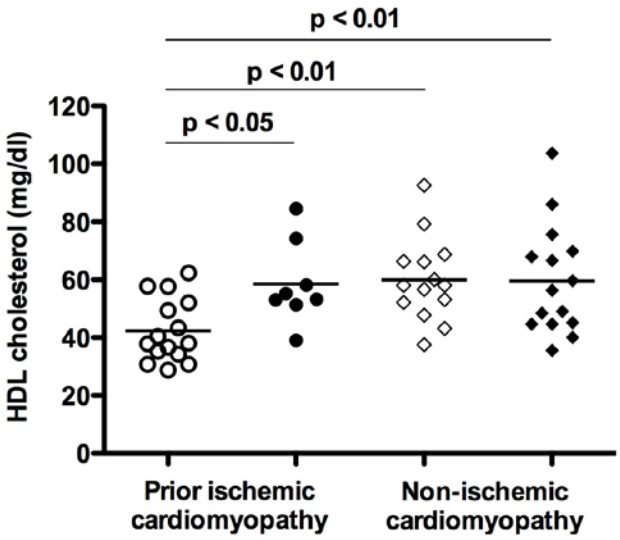

Figure 3.3.3. Individual value bar graph of HDL cholesterol levels in patients with prior ischemic cardiomyopathy (circles) and in patients with prior non-ischemic cardiomyopathy (diamonds). Open and closed symbols represent patients not taking steroids and patients taking steroids, respectively. Data points show the individual values. Means are shown by the horizontal lines.

Table 3.3.5. Comparison of lipoprotein parameters, HDL function and CRP level between patients on steroid and patients not on steroid.

	Patients with steroid (n=23)	Patients without steroid (n=29)	P value
Age at inclusion in the study (years)	62.5 ± 2.9	60.3 ± 2.6	0.558
Sex (male/female)	16 (69.6%)/7 (30.4%)	26 (89.7%)/3 (10.3%)	0.0866
Cholesterol (mg/dl)	167 ± 8	156 ± 6	0.267
Triglycerides (mg/dl)	129 ± 15	108 ± 6	0.185
HDL cholesterol (mg/dl)	59.2 ± 3.6	50.8 ± 2.8	0.0552
LDL cholesterol (mg/dl)	82.7 ± 4.7	83.7 ± 5.0	0.895
Triglyceride/HDL cholesterol ratio	2.35 ± 0.32	2.37 ± 0.20	0.944
Triglyceride/HDL cholesterol ratio ≥ 3	4 (17.4%)	9 (31.0%)	0.341
Apolipoprotein A-I (mg/dl)	128 ± 7	116 ± 5	0.175
CRP (mg/l)	13.0 ± 4.2	7.2 ± 1.4	0.158
CRP ≥ 6 mg/l	11(47.8%)	11(37.9%)	0.576
Normalized cholesterol efflux	0.853± 0.049	0.781 ± 0.053	0.338
Vasculoprotective function of HDL (migrated cells/mm^2)	399 ± 23	426 ± 24	0.424

Data are expressed as means ± SEM for continuous variables.

3.3.4.7 Distinct immunosuppressive regimens do not differentially affect HDL function

The majority of patients in the current study were being treated with the combination tacrolimus/mycophenolate mofetil (with or without steroids), which is the current standard immunosuppressive regimen in most centers. We compared different parameters in heart transplant recipients categorized according to immunosuppression with or without tacrolimus (Table 3.3.6) and according to immunosuppression with or without myophenolate mofetil (Table 3.3.7). HDL cholesterol levels, apo A-I levels, cholesterol efflux capacity of HDL, and vasculoprotective function of HDL were similar in patients with or without tacrolimus immunosuppression (Table 3.3.6) and in patients with or without myophenolate mofetil immunosuppression (Table 3.3.7). The lower triglyceride level in patients on tacrolimus (Table 3.3.6) is in agreement with data of randomized trials[24, 25].

Furthermore, compared to reference values in healthy controls, the cholesterol efflux capacity of HDL (1.071 $\pm$ 0.059 versus 0.827 $\pm$ 0.047; p=0.0018) and the vasculoprotective function of HDL (526 $\pm$ 33 versus 404 $\pm$ 21; p=0.0018) were impaired in heart transplant recipients taking tacrolimus. Similarly, the cholesterol efflux capacity of HDL (1.071 $\pm$ 0.059 versus 0.829 $\pm$ 0.047; p=0.0022) and the vasculoprotective function of HDL (526 $\pm$ 33 versus 419 $\pm$ 20; p=0.0048) were lower in patients treated with myophenolate mofetil compared to healthy controls. Therefore, the impairment of cholesterol efflux capacity and of the vasculoprotective function of HDL in heart transplant recipients appears to be independent of the specific immunosuppressive regimen. However, this does not exclude that immunosuppressive drugs may be directly implicated in impaired HDL function.

Table 3.3.6. Comparison of lipoprotein parameters, HDL function and CRP level between patients on tacrolimus and patients not on tacrolimus.

	Patients with tacrolimus (n=35)	Patients without tacrolimus (n=17)	P value
Age at inclusion in the study (years)	59.7 ± 2.4	64.4 ± 3.1	0.249
Sex (male/female)	25 (71.4%)/10 (28.6%)	17 (100%)/0 (0%)	0.0206
Cholesterol (mg/dl)	159 ± 6	165 ± 9	0.599
Triglycerides (mg/dl)	106 ± 6	141 ± 20	0.0333
HDL cholesterol (mg/dl)	56.5 ± 3.0	50.6 ± 3.0	0.236
LDL cholesterol (mg/dl)	81.6 ± 4.5	86.6 ± 5.2	0.498
Triglyceride/HDL cholesterol ratio	2.09 ± 0.17	2.93 ± 0.40	0.0259
Triglyceride/HDL cholesterol ratio ≥ 3	6 (17.1%)	7 (41.2%)	0.0890
Apolipoprotein A-I (mg/dl)	124 ± 5	116 ± 7	0.389
CRP (mg/l)	9.24 ± 2.28	10.8 ± 4.2	0.721
CRP ≥ 6 mg/l	14 (40.0%)	7 (41.2%)	1.00
Normalized cholesterol efflux	0.827± 0.047	0.783 ± 0.059	0.571
Vasculoprotective function of HDL (migrated cells/mm^2)	404 ± 21	435 ± 28	0.397

Data are expressed as means ± SEM for continuous variables.

Table 3.3.7. Comparison of lipoprotein parameters, HDL function and CRP level between patients on mycophenolate mofetil and patients not on mycophenolate mofetil.

	Patients with mycophenolate mofetil (n=37)	Patients without mycophenolate mofetil (n=15)	P value
Age at inclusion in the study (years)	59.6 ± 2.2	65.2 ± 3.5	0.182
Sex (male/female)	30 (81.1%)/7 (18.9%)	12 (80.0%)/3 (20.0%)	1.00
Cholesterol (mg/dl)	163 ± 6	156 ± 8	0.481
Triglycerides (mg/dl)	116 ± 10	122 ± 10	0.738
HDL cholesterol (mg/dl)	55.3 ± 2.9	52.7 ± 3.6	0.613
LDL cholesterol (mg/dl)	85.0 ± 4.4	79.0 ± 4.9	0.432
Triglyceride/HDL cholesterol ratio	2.33 ± 0.23	2.45 ± 0.26	0.754
Triglyceride/HDL cholesterol ratio ≥ 3	9 (24.3%)	4 (26.7%)	1.00
Apolipoprotein A-I (mg/dl)	122 ± 4	121 ± 10	0.990
CRP (mg/l)	9.25 ± 2.06	11.0 ± 5.0	0.703
CRP ≥ 6 mg/l	17 (46.0%)	5 (33.3%)	0.539
Normalized cholesterol efflux	0.829± 0.047	0.773 ± 0.049	0.497
Vasculoprotective function of HDL (migrated cells/mm^2)	419 ± 20	402 ± 31	0.655

Data are expressed as means ± SEM for continuous variables.

3.3.5 DISCUSSION

The salient findings of the present study are that 1) notwithstanding similar HDL cholesterol levels in heart transplant recipients compared to healthy controls, HDL function measured by two highly distinct assays is decreased in heart transplant patients; 2) HDL function is not different between CAV-negative and CAV-positive patients; 3) the proportion of patients with a CRP level equal to or greater than 6 mg/l is prominently higher in CAV-positive patients.

HDL cholesterol levels were similar in heart transplant recipients compared to healthy controls. The healthy controls contained a higher proportion of female subjects and were younger compared to heart transplant patients. In prospective studies that are not affected by survival bias, HDL cholesterol levels decrease with age in men and women[26]. Irrespective of potential sources of bias in this comparison, we focused on potential determinants of HDL cholesterol levels in these patients. Our *a priori* hypothesis was that HDL cholesterol levels would be influenced by corticosteroid use and by heart failure etiology before transplantation. In line of known effects of corticosteroids on HDL metabolism and HDL cholesterol levels[12], HDL cholesterol levels were higher in patients on corticosteroids compared to patients on a steroid-free immunosuppressive regimen. On the other hand, HDL cholesterol levels were significantly lower in patients with prior ischemic cardiomyopathy than in patients with prior non-ischemic cardiomyopathy. This cannot be explained by the worse renal function or by the slightly higher age in patients transplanted for ischemic cardiomyopathy. Since low HDL cholesterol is an independent risk marker for coronary artery disease[27], lower HDL cholesterol concentrations in these patients after heart transplantation probably reflect the pretransplant status. Unexpectedly, an interaction was observed between etiology of heart failure before transplantation and steroid use as determinants of HDL cholesterol levels. Taken together, the absence of a difference of HDL cholesterol levels between heart transplant recipients and healthy controls should be interpreted in light of the interaction between these two factors.

Because HDL particles carry more than 80 different proteins and more than 200 lipid species as well as several microRNAs[28], the structural and functional complexity of HDL cannot be captured by a simple biochemical HDL metric such as HDL cholesterol. Therefore, we evaluated two highly distinct dimensions of HDL function: the cholesterol efflux capacity and the vasculoprotective function of HDL as analyzed by an EPC migration assay. A marked decline of HDL function was observed in heart transplant recipients but no difference in HDL function occurred according to CAV-status. Furthermore, HDL function was not affected by corticosteroid use and was not different in patients with prior ischemic cardiomyopathy compared to prior non-ischemic cardiomyopathy. De la Llera-Moya *et al*.[29] have previously demonstrated that the cholesterol efflux capacity of apo B-depleted serum is significantly correlated with the concentration of pre-ß1-HDL. Serum pre-ß1-HDL depends on the activity of phospholipid transfer protein, hepatic lipase, and cholesterol ester transfer protein[30, 31]. Pre-ß1-HDL is significantly lower in heart transplant recipients than in healthy controls[11], which is likely related to the lower activities of these three enzymes. Lower pre-ß1-HDL may be one factor that underlies the lower cholesterol efflux capacity

of apo B-depleted sera of heart transplant recipients. In general, HDL dysfunction may be due to post-translational modifications of proteins or to compositional changes of the proteome or the lipidome[28]. Importantly, the correlation between cholesterol efflux capacity and vasculoprotective function of HDL was weak, indicating that the attenuation of both dimensions of HDL function in heart transplant recipients reflect distinct biochemical alterations of HDL. The impaired stimulation of murine EPC migration by HDL from heart transplant recipients is consistent with impaired migration of endogenous EPCs in these patients[19].

In two prospective studies, CRP levels and the triglyceride/HDL cholesterol ratio, considered to be a marker of insulin resistance, were independent risk markers for CAV and major adverse cardiac events[8, 9]. In the current cross-sectional study, we observed that the proportion of patients with a CRP level greater than or equal to 6 mg/l was higher in CAV-positive patients than in CAV-negative patients. However, the percentage of patients with a triglyceride/HDL cholesterol ratio greater than or equal to 3 was not significantly higher in the CAV-positive group. Although this may be related to a lack of statistical power, this may also be related to the lower body mass index of patients in the current study compared to prior studies[14, 15]. Furthermore, whether the proposed cut-off value of 3 of this parameter is a sensitive and specific marker of insulin resistance has not been validated in heart transplant recipients. Considering the likely impact of steroids on HDL cholesterol levels and on insulin sensitivity, the interpretation of the triglyceride/HDL cholesterol ratio in heart transplant recipients may be cumbersome.

In conclusion, HDL function measured by two highly distinct assays is decreased in heart transplant patients. HDL dysfunction may be related to alterations of HDL metabolism in these patients or to undefined compositional alterations that have an overriding negative impact.

3.3.6 REFERENCES

1. Schmauss, D. ,Weis, M. Cardiac allograft vasculopathy: recent developments. *Circulation* **117**, 2131-2141 (2008).
2. Rahmani, M., Cruz, R.P., Granville, D.J. ,McManus, B.M. Allograft vasculopathy versus atherosclerosis. *Circ Res* **99**, 801-815 (2006).
3. Parameshwar, J., Foote, J., Sharples, L., Wallwork, J., Large, S. ,Schofield, P. Lipids, lipoprotein (a) and coronary artery disease in patients following cardiac transplantation. *Transpl Int* **9**, 481-485 (1996).
4. Valantine, H., Rickenbacker, P., Kemna, M., Hunt, S., Chen, Y.D., Reaven, G. ,Stinson, E.B. Metabolic abnormalities characteristic of dysmetabolic syndrome predict the development of transplant coronary artery disease: a prospective study. *Circulation* **103**, 2144-2152 (2001).
5. Cooke, G.E., Eaton, G.M., Whitby, G., Kennedy, R.A., Binkley, P.F., Moeschberger, M.L. ,Leier, C.V. Plasma atherogenic markers in congestive heart failure and posttransplant (heart) patients. *J Am Coll Cardiol* **36**, 509-516 (2000).
6. Sanchez-Gomez, J.M., Martinez-Dolz, L., Sanchez-Lazaro, I., Almenar, L., Sanchez-Lacuesta, E., Munoz-Giner, B., Portoles, M., Rivera, M., Valera-Roman, A., Gonzalez-Juanatey, J.R., Tejada-Ponce, D., Aguero, J., Buendia, F. ,Salvador, A. Influence of metabolic syndrome on development of cardiac allograft vasculopathy in the transplanted heart. *Transplantation* **93**, 106-111 (2012).
7. McLaughlin, T., Abbasi, F., Cheal, K., Chu, J., Lamendola, C. ,Reaven, G. Use of metabolic markers to identify overweight individuals who are insulin resistant. *Ann Intern Med* **139**, 802-809 (2003).
8. Biadi, O., Potena, L., Fearon, W.F., Luikart, H.I., Yeung, A., Ferrara, R., Hunt, S.A., Mocarski, E.S. ,Valantine, H.A. Interplay between systemic inflammation and markers of insulin resistance in cardiovascular prognosis after heart transplantation. *J Heart Lung Transplant* **26**, 324-330 (2007).
9. Raichlin, E.R., McConnell, J.P., Lerman, A., Kremers, W.K., Edwards, B.S., Kushwaha, S.S., Clavell, A.L., Rodeheffer, R.J. ,Frantz, R.P. Systemic inflammation and metabolic syndrome in cardiac allograft vasculopathy. *J Heart Lung Transplant* **26**, 826-833 (2007).
10. Atger, V., Leclerc, T., Cambillau, M., Guillemain, R., Marti, C., Moatti, N. ,Girard, A. Elevated high density lipoprotein concentrations in heart transplant recipients are related to impaired plasma cholesteryl ester transfer and hepatic lipase activity. *Atherosclerosis* **103**, 29-41 (1993).
11. Sviridov, D., Chin-Dusting, J., Nestel, P., Kingwell, B., Hoang, A., Olchawa, B., Starr, J. ,Dart, A. Elevated HDL cholesterol is functionally ineffective in cardiac transplant recipients: evidence for impaired reverse cholesterol transport. *Transplantation* **81**, 361-366 (2006).

12. Stamler, J.S., Vaughan, D.E. ,Loscalzo, J. Immunosuppressive therapy and lipoprotein abnormalities after cardiac transplantation. *Am J Cardiol* **68**, 389-391 (1991).
13. Yamashita, S., Tsubakio-Yamamoto, K., Ohama, T., Nakagawa-Toyama, Y. ,Nishida, M. Molecular mechanisms of HDL-cholesterol elevation by statins and its effects on HDL functions. *J Atheroscler Thromb* **17**, 436-451 (2010).
14. Fisher, E.A., Feig, J.E., Hewing, B., Hazen, S.L. ,Smith, J.D. High-density lipoprotein function, dysfunction, and reverse cholesterol transport. *Arterioscler Thromb Vasc Biol* **32**, 2813-2820 (2012).
15. Khera, A.V., Cuchel, M., de la Llera-Moya, M., Rodrigues, A., Burke, M.F., Jafri, K., French, B.C., Phillips, J.A., Mucksavage, M.L., Wilensky, R.L., Mohler, E.R., Rothblat, G.H. ,Rader, D.J. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *N Engl J Med* **364**, 127-135 (2011).
16. Vassalli, G., Gallino, A., Weis, M., von Scheidt, W., Kappenberger, L., von Segesser, L.K. ,Goy, J.J. Alloimmunity and nonimmunologic risk factors in cardiac allograft vasculopathy. *Eur Heart J* **24**, 1180-1188 (2003).
17. Smadja, D.M., Bieche, I., Uzan, G., Bompais, H., Muller, L., Boisson-Vidal, C., Vidaud, M., Aiach, M. ,Gaussem, P. PAR-1 activation on human late endothelial progenitor cells enhances angiogenesis in vitro with upregulation of the SDF-1/CXCR4 system. *Arterioscler Thromb Vasc Biol* **25**, 2321-2327 (2005).
18. Feng, Y., Jacobs, F., Van Craeyveld, E., Brunaud, C., Snoeys, J., Tjwa, M., Van Linthout, S. ,De Geest, B. Human ApoA-I transfer attenuates transplant arteriosclerosis via enhanced incorporation of bone marrow-derived endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* **28**, 278-283 (2008).
19. Singh, N., Van Craeyveld, E., Tjwa, M., Ciarka, A., Emmerechts, J., Droogne, W., Gordts, S.C., Carlier, V., Jacobs, F., Fieuws, S., Vanhaecke, J., Van Cleemput, J. ,De Geest, B. Circulating apoptotic endothelial cells and apoptotic endothelial microparticles independently predict the presence of cardiac allograft vasculopathy. *J Am Coll Cardiol* **60**, 324-331 (2012).
20. Mehra, M.R., Crespo-Leiro, M.G., Dipchand, A., Ensminger, S.M., Hiemann, N.E., Kobashigawa, J.A., Madsen, J., Parameshwar, J., Starling, R.C. ,Uber, P.A. International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for cardiac allograft vasculopathy-2010. *J Heart Lung Transplant* **29**, 717-727 (2010).
21. Asztalos, B.F., de la Llera-Moya, M., Dallal, G.E., Horvath, K.V., Schaefer, E.J. ,Rothblat, G.H. Differential effects of HDL subpopulations on cellular ABCA1- and SR-BI-mediated cholesterol efflux. *J Lipid Res* **46**, 2246-2253 (2005).
22. Khera, A.V., Cuchel, M., de la Llera-Moya, M., Rodrigues, A., Burke, M.F., Jafri, K., French, B.C., Phillips, J.A., Mucksavage, M.L., Wilensky, R.L., Mohler, E.R., Rothblat, G.H. ,Rader, D.J. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *N Engl J Med* **364**, 127-135 (2011).

23. Van Linthout, S., Collen, D. ,De Geest, B. Effect of promoters and enhancers on expression, transgene DNA persistence, and hepatotoxicity after adenoviral gene transfer of human apolipoprotein A-I. *Hum Gene Ther* **13**, 829-840. (2002).
24. Kobashigawa, J.A., Miller, L.W., Russell, S.D., Ewald, G.A., Zucker, M.J., Goldberg, L.R., Eisen, H.J., Salm, K., Tolzman, D., Gao, J., Fitzsimmons, W. ,First, R. Tacrolimus with mycophenolate mofetil (MMF) or sirolimus vs. cyclosporine with MMF in cardiac transplant patients: 1-year report. *Am J Transplant* **6**, 1377-1386 (2006).
25. Taylor, D.O., Barr, M.L., Radovancevic, B., Renlund, D.G., Mentzer, R.M., Jr., Smart, F.W., Tolman, D.E., Frazier, O.H., Young, J.B. ,VanVeldhuisen, P. A randomized, multicenter comparison of tacrolimus and cyclosporine immunosuppressive regimens in cardiac transplantation: decreased hyperlipidemia and hypertension with tacrolimus. *J Heart Lung Transplant* **18**, 336-345 (1999).
26. Ferrara, A., Barrett-Connor, E. ,Shan, J. Total, LDL, and HDL cholesterol decrease with age in older men and women. The Rancho Bernardo Study 1984-1994. *Circulation* **96**, 37-43 (1997).
27. Di Angelantonio, E., Sarwar, N., Perry, P., Kaptoge, S., Ray, K.K., Thompson, A., Wood, A.M., Lewington, S., Sattar, N., Packard, C.J., Collins, R., Thompson, S.G. ,Danesh, J. Major lipids, apolipoproteins, and risk of vascular disease. *Jama* **302**, 1993-2000 (2009).
28. Annema, W. ,von Eckardstein, A. High-density lipoproteins. Multifunctional but Vulnerable Protections from Atherosclerosis. *Circ J* **77**, 2432-2448 (2013).
29. de la Llera-Moya, M., Drazul-Schrader, D., Asztalos, B.F., Cuchel, M., Rader, D.J. ,Rothblat, G.H. The ability to promote efflux via ABCA1 determines the capacity of serum specimens with similar high-density lipoprotein cholesterol to remove cholesterol from macrophages. *Arterioscler Thromb Vasc Biol* **30**, 796-801 (2010).
30. Zhou, H., Tan, K.C., Shiu, S.W. ,Wong, Y. Cellular cholesterol efflux to serum is impaired in diabetic nephropathy. *Diabetes Metab Res Rev* **24**, 617-623 (2008).
31. O'Connor, P.M., Zysow, B.R., Schoenhaus, S.A., Ishida, B.Y., Kunitake, S.T., Naya-Vigne, J.M., Duchateau, P.N., Redberg, R.F., Spencer, S.J., Mark, S., Mazur, M., Heilbron, D.C., Jaffe, R.B., Malloy, M.J. ,Kane, J.P. Prebeta-1 HDL in plasma of normolipidemic individuals: influences of plasma lipoproteins, age, and gender. *J Lipid Res* **39**, 670-678 (1998).

3.4 ENDOTHELIUM-ENRICHED MICRORNAs PREDICT THE PRESENCE OF CARDIAC ALLOGRAFT VASCULOPATHY*

3.4.1 ABSTRACT

Background: Cardiac allograft vasculopathy (CAV) is a limiting factor for the long-term survival of heart transplant recipients. Clinical decisions and care may be improved by the development of prediction models based on circulating biomarkers. The endothelium may play a central pathogenetic role in the development of CAV. We evaluated the hypothesis that endothelium-enriched microRNAs (miRNAs) discriminate between patients with CAV and patients without CAV.

Methods: Fifty-two patients undergoing coronary angiography between 5 and 15 years after heart transplantation were recruited in this cross-sectional study. Circulating levels of endothelium-enriched miRNAs (miR-21-5p, miR-92a-3p, miR-92a-1-5p, miR-126-3p, miR-126-5p) were quantified by real-time RT-PCR. The discriminative ability of logistic regression models was evaluated using the concordance statistic (c-statistic).

Results: Median plasma levels of miR-21-5p, miR-92a-3p, miR-126-3p, and miR-126-5p were 1.82-fold (p=NS), 1.87-fold (p<0.05), 1.94-fold (p=0.074), and 1.59-fold (p=0.060) higher, in patients with CAV than in patients without CAV. Recipient age (c-statistic 0.689 (95% CI 0.537-0.842)), serum creatinine (c-statistic 0.703 (95% CI 0.552-0.854)), levels of miR-92a-3p (c-statistic 0.682 (95% CI 0.533-0.831)), and levels of miR-126-5p (c-statistic 0.655 (95% CI 0.502-0.807)) predicted CAV-status in univariable models. In multivariable logistic regression models with recipient age and creatinine as covariates, miR-126-5p (χ^2=4.37; df=1; p=0.037), miR-92a-3p (χ^2=6.01; df=1; p=0.014), and the combination of miR-126-5p and miR-92a-3p (χ^2=8.16; df=2; p=0.017) added significant information. The model with age, creatinine, miR-126-5p, and miR-92a-3p as covariables conferred good discrimination between patients without CAV and patients with CAV (c-statistic 0.800 (95% CI 0.674-0.926)).

Conclusion: Endothelium-enriched miRNAs have predictive ability for CAV beyond clinical predictors.

3.4.2 INTRODUCTION

Cardiac allograft vasculopathy (CAV) is a limiting factor for the long-term survival of heart transplant recipients[1,2]. CAV is characterized by the development of diffuse concentric fibromuscular intimal hyperplasia lesions in epicardial and smaller intramyocardial arteries along with focal, eccentric atherosclerotic plaques in the larger epicardial arteries[3,4]. The development of these lesions may lead to the progressive narrowing of the lumen[5]. According to the response to injury hypothesis of CAV, these lesions are the result of cumulative endothelial injury induced by

*This chapter has been submitted as: **Singh, N.**, Heggermont, W., Fieuws, S., Vanhaecke, J., Van Cleemput, J., De Geest, B. Endothelium-enriched microRNAs predict the presence of cardiac allograft vasculopathy. 2015.

alloimmune responses as well as non-immunological risk factors such as ischemia-reperfusion injury, viral infections, and metabolic disorders[3,6].

Early diagnosis of CAV is essential to implement appropriate prevention and treatment measures. Metabolic parameters like triglycerides to HDL cholesterol ratio[7,8] and plasma insulin level[9] may discriminate between CAV-positive and CAV-negative patients. Immunological and inflammatory biomarkers of CAV include donor-specific anti-HLA antibodies[10], antibodies against heterogeneous nuclear ribonucleoprotein K[11], C-reactive protein (CRP)[8,12-14], vascular cell adhesion molecule-1[15], and circulating C-X-C motif chemokine 12 (CXCL12) levels[16]. However, the discriminative ability and the incremental value of these biomarkers beyond clinical risk factors have not been robustly established. Candidate-based approaches using biomarkers of endothelial homeostasis may constitute a solid foundation for the development of prediction models of CAV. The angiogenesis-related proteins vascular endothelial growth factor (VEGF)-C, VEGF-A and platelet factor-4 have been identified as independent biomarkers of CAV[17]. In a recent cross-sectional study[18], we demonstrated that a logistic regression model containing apoptotic circulating endothelial cells (CECs) and apoptotic circulating endothelial microparticles (CEMPs) as independent predictors provided high discrimination between CAV-positive and CAV-negative patients (c-statistic 0.812; 95% CI 0.692-0.932). In several logistic regression models including clinical and biochemical covariates, the introduction of apoptotic CECs and apoptotic CEMPs consistently resulted in added value, indicating that these biomarkers are robust independent predictors.

In line with previous studies demonstrating the ability of biomarkers related to endothelial homeostasis to predict CAV, the aim of the current study was to analyze the potential of endothelium-enriched microRNAs (miRNAs) as putative biomarkers for the prediction of CAV. MiRNAs are small, non-coding, single-stranded RNA sequences that regulate gene expression at the post-transcriptional level. Because miRNAs circulate in remarkably stable forms in blood[19,20], they have a significant potential as biomarkers. Several reports indicate that miRNAs may play a role in endothelial homeostasis[21,22]. In this study, a candidate-based approach using circulating levels of endothelium-enriched miRNAs (miR-21-5p, miR-92a-3p, miR-92a-1-5p, miR-126-3p, miR-126-5p) to predict CAV was investigated.

3.4.3 METHODS

3.4.3.1 Study design

Fifty-two clinically stable patients undergoing coronary angiography between 5 and 15 years after heart transplantation were included in this cross-sectional study. In addition, eighty patients with clinically stable native coronary artery disease (CAD) were recruited in the study. Stable native CAD patients were defined by the presence of at least one stenosis of 50% or more demonstrated by diagnostic coronary angiography. CAV was graded according to the International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for CAV-2010[23]. The clinical characteristics of CAV-negative and CAV-positive patients have been

described in a previous report[18]. Heart transplant patients with prior congenital heart disease and re-transplanted patients were excluded. The study was approved by the Ethics Committee of the University Hospital Gasthuisberg and written informed consent was obtained from all participating subjects. The reference control group included 25 healthy control subjects (12 males and 13 females) with an average age of 43.2 ± 2.0 years[24].

3.4.3.2 Quantification of circulating levels of endothelium-enriched miRNAs (miR-21-5p, miR-92a-3p, miR-92a-1-5p, miR-126-3p, miR-126-5p) in plasma samples

This study was not preceded by a screening phase evaluating a large pool of miRNAs for association with CAV-status. The endothelium-enriched miRNAs (miR-21, miR-92a, miR-126) investigated in this study were *a priori* selected based on an analysis of the literature and no other miRNAs were quantified. Peripheral blood was drawn by venipuncture using Vacutainer® collection tubes (BD Diagnostics, Franklin Lakes, NJ, USA). Plasma derived from EDTA anticoagulated peripheral blood was centrifuged within one hour after collection at 1900 g for 10 min followed by a second centrifugation at 1900 g for 20 min to generate platelet-poor plasma (PPP) that was used for miRNA quantification by real-time PCR. RNA was isolated from 400µl plasma using the Ambion mirVANA RNA extraction kit (AM1560, Applied Biosciences, Austin, Texas, USA) according to the instructions of the manufacturer. Subsequently, 300 nanogram of RNA was reverse-transcribed to cDNA using the miScript-II RT PCR kit (218061, Qiagen Benelux NV, Venlo, The Netherlands) according to the manufacturer's protocol. Real-time PCR was performed on an ABI-Prism cycler (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) using LNA™-based miRNA primers (Exiqon A/S, Vedbaek, Denmark) and SYBR Green (Life Technologies, Carlsbad, CA, USA). U6 non-coding small nuclear RNA (snRNA) expression was measured as an endogenous control for data normalization. U6 primers were designed by Eurogentec (Eurogentec, Seraing, Belgium). MiRNA expression levels were compared using the relative threshold cycle (Ct) method ($2^{-\Delta\Delta Ct}$).

3.4.3.3 Statistical analysis

Clinical and biochemical parameters and endothelial biomarkers were compared using Instat 3 (Graphpad software, San Diego, CA, USA). Continuous variables were summarized by means, standard error of the mean, and sample size, and were compared by Student t-test between CAV-negative and CAV-positive patients and between CAV-positive patients and stable native CAD patients. When data were not normally distributed, data are presented as medians and interquartile range (IQR), and were compared by a Mann-Whitney Test. Logistic regression analysis was performed by SAS software, version 9.2 (SAS Institute Inc., Cary, NC, USA). Since the distribution of the concentration of CECs and of CRP is heavily right-skewed, a natural logarithm transformation of the data of these two parameters was applied for logistic regression analysis. The discriminative ability was quantified using the concordance statistic (C-statistic), which is equal to the area under the receiver operating characteristic curve. Evaluation of added

value of predictors in multivariable logistic regression models was based on likelihood-ratio tests comparing two nested models. A p-value of less than 0.05 was considered statistically significant.

3.4.4 RESULTS

3.4.4.1 Characteristics of heart transplant recipients without and with CAV and of patients with stable native CAD

The clinical characteristics of heart transplant recipients without CAV (n=22) and with CAV (n=30), and of patients with stable native CAD (n=80) are shown in Table 3.4.1. Patients with CAV were 8.6 years ($p<0.05$) older than patients without CAV and 4.7 years ($p<0.05$) younger than patients with stable native CAD at the time of inclusion in the study. Patients with stable native CAD had a lower prevalence of hypertension ($p<0.0001$) and a higher body mass index ($p<0.05$) compared to patients with CAV. No significant difference of these two parameters was observed between patients without CAV and patients with CAV. Lipoprotein levels were similar among the three patient groups except LDL cholesterol, which was 15.9% ($p<0.05$) lower in patients with CAV than in patients without CAV. Creatinine levels were significantly higher in patients with CAV than in patients without CAV ($p<0.05$) as well as in patients with stable native CAD ($p<0.0001$). There was a trend ($p=0.082$) for higher CRP levels in patients with CAV compared to patients without CAV. Median CRP was 4.33-fold ($p<0.0001$) higher in patients with CAV than in patients with stable native CAD. Statin use was generalized in all the three patient groups.

Table 3.4.1. Clinical characteristics, laboratory parameters, immunosuppressive and hypolipidemic therapy of patients without CAV, patients with CAV and of patients with stable native CAD.

	Patients without CAV (n=22)	Patients with CAV (n=30)	Patients with stable native CAD (n=80)
Age at inclusion in the study (years)	56.3 ± 3.2	64.9 ± 2.1*	69.6 ± 1.2†
Sex (male/female)	81.8%/18.2%	80.0%/20.0%	78.8%/21.3%
Donor age (years)	33.8 ± 2.9	40.8 ± 2.4	N.A.
Time after heart transplantation (years)	9.35 ± 0.58	10.9 ± 0.8	N.A.
Age of the transplanted heart (years)	43.2 ± 3.0	51.7 ± 2.3*	N.A.
Sex mismatch graft (%)	13.6%	20.0%	N.A.
Acute rejection episodes (Grade 3A or > 3A)	13.6%	10.0%	N.A.
Current smoker (%)	13.6%	0%	7.5%
Hypertension (%)	90.9%	100%	47.5%††††
Diabetes (%)	18.2%	36.7%	25.0%
Body mass index (kg/m^2)	26.6 ± 0.8	25.5 ± 0.5	27.5 ± 0.6†
Cholesterol (mg/dl)	168 ± 8	156 ± 6	160 ± 4
Triglycerides (mg/dl)	108 ± 7	125 ± 12	132 ± 7
HDL cholesterol (mg/dl)	55.0 ± 2.9	54.2 ± 3.4	50.2 ± 1.8
LDL cholesterol (mg/dl)	91.6 ± 5.7	77.1 ± 4.0*	83.6 ± 3.0
Creatinine (mg/dl)	1.32 ± 0.10	1.60 ± 0.08*	1.05 ± 0.03††††
CRP (mg/l)	2.99 (1.85-6.65)	6.71 (3.25-12.0)	1.55 (0.60-2.99)††††
Statins (%)	95.5%	100%	95.0%
Cyclosporine (%)	31.8%	23.3%	N.A.
Tacrolimus (%)	63.6%	70.0%	N.A.
Everolimus (%)	4.55%	20.0%	N.A.
Azathioprine (%)	9.10%	13.3%	N.A.
Mycophenolate mofetil (%)	86.4%	60.0%	N.A.
Steroid (%)	18.2%	63.3%**	N.A.

Data of continuous variables represent means ± SEM or median (IQR).

*** : p<0.05, ** : p<0.01, and *** : p<0.001 for patients with CAV versus patients without CAV.**

† : p<0.05, †† : p<0.01, ††† : p<0.001, and †††† : p<0.0001 for patients with stable native CAD versus patients with CAV.

N.A.: not applicable.

3.4.4.2 Circulating endothelium-enriched miRNA levels are higher in patients with CAV compared to patients without CAV

All miRNA levels (Figure 3.4.1, Figure 3.4.2) were normalized against U6 snRNA level. As shown in Figure 3.4.1A, median plasma miR-21-5p level was 2.34-fold ($p<0.05$) increased in patients with CAV compared to healthy controls whereas no increase was observed in CAV-negative patients. Median plasma miR-21-5p level was 1.82-fold (p=NS) higher in CAV-positive patients compared to CAV-negative patients (Figure 3.4.1A). Median plasma miR-92a-3p in patients with CAV was 3.08-fold ($p<0.01$) and 1.87-fold ($p<0.05$) higher, respectively, compared to healthy controls and patients without CAV (Figure 3.4.1B). As shown in Figure 3.4.1C, median plasma miR-92a-1-5p was 2.11-fold increased ($p=0.075$) in patients with CAV compared to healthy controls but no significant difference was observed between CAV-negative and CAV-positive patients. Median plasma miR-126-3p in CAV-positive patients was 2.80-fold ($p<0.01$) and 1.94-fold ($p=0.074$) higher, respectively, compared to healthy controls and CAV-negative patients. Finally, median plasma miR-126-5p in patients with CAV was 2.02-fold ($p<0.05$) and 1.59-fold ($p=0.060$) increased, respectively, compared to healthy controls and patients without CAV. Taken together, these results demonstrate that levels of several circulating endothelium-enriched miRNAs are increased in patients with CAV compared to patients without CAV.

3.4.4.3 Circulating levels of miR-92a-3p and miR-92a-1-5p differ in patients with CAV and in patients with stable native CAD

Median plasma levels of miR-21-5p, miR-92a-3p, miR-92a-1-5p, miR-126-3p, and miR-126-5p were 2.38-fold ($p<0.01$), 1.98-fold ($p<0.01$), 3.16-fold ($p<0.0001$), 3.10-fold ($p<0.0001$), and 1.90-fold ($p<0.0001$) higher, respectively, in patients with stable native CAD (Figure 3.4.2) than in healthy controls (Figure 3.4.1). Whereas all of these 5 endothelium-enriched miRNAs were elevated in both CAV and native CAD, two distinctions in miRNA levels were observed between these two types of arteriosclerosis. Median plasma level of miR-92a-3p was elevated 1.56-fold ($p=0.051$) in CAV-positive patients compared to patients with stable native CAD (Figure 3.4.2B). In contrast, median plasma level of miR-92a-1-5p was 1.50-fold ($p=0.089$) higher in patients with native CAD compared to patients with CAV (Figure 3.4.2C).

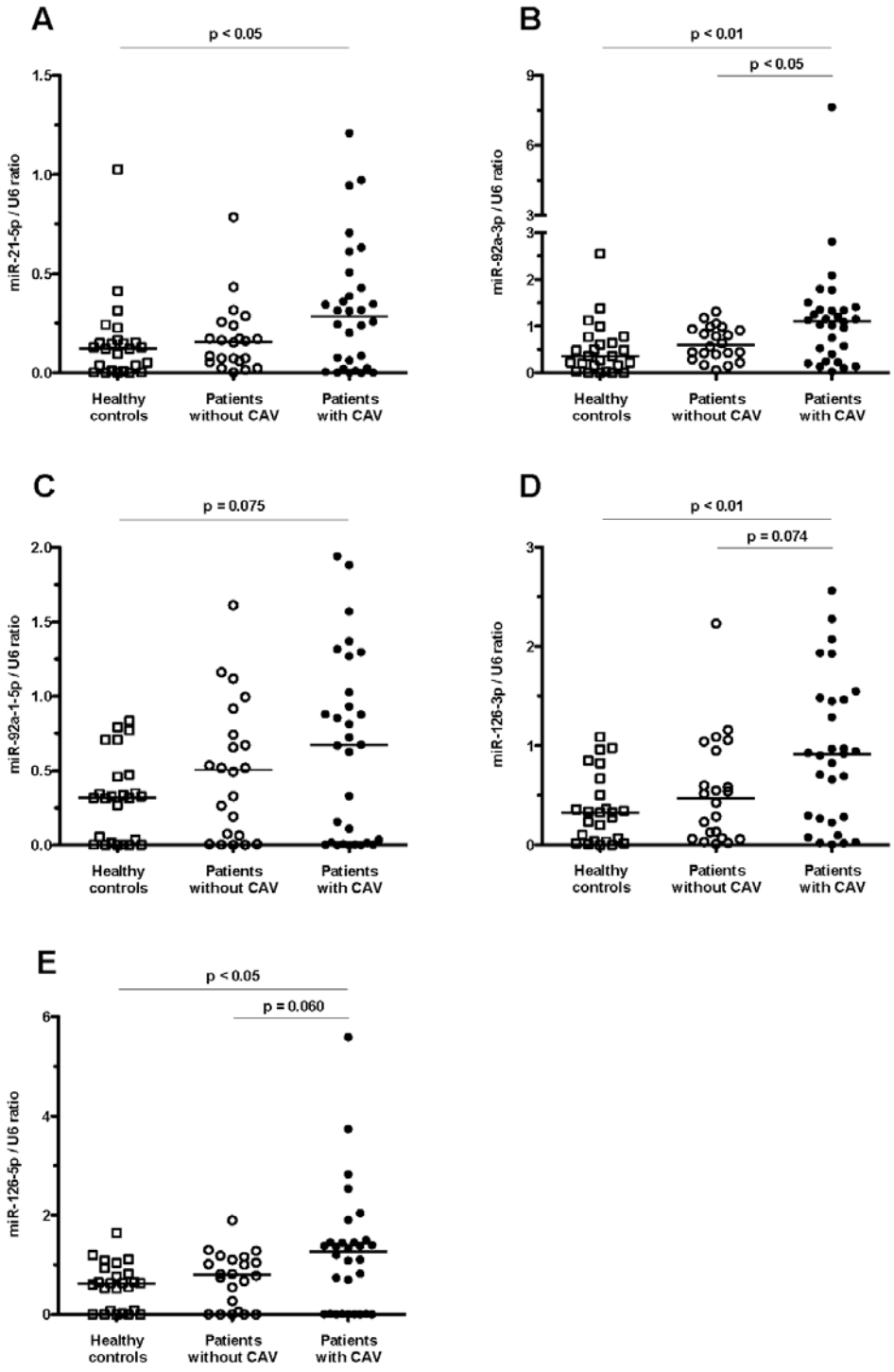

Figure 3.4.1. Individual value bar graph illustrating a comparison of plasma level of miR-21-5p (panel A), miR-92a-3p (panel B), miR-92a-1-5p (panel C), miR-126-3p (panel D), and miR-126-5p (panel E) in healthy controls (n=25), patients without CAV (n=22), and patients with CAV (n=30). All miRNA levels were normalized against U6 snRNAlevel. Data points show the individual values. Medians are shown by the horizontal lines.

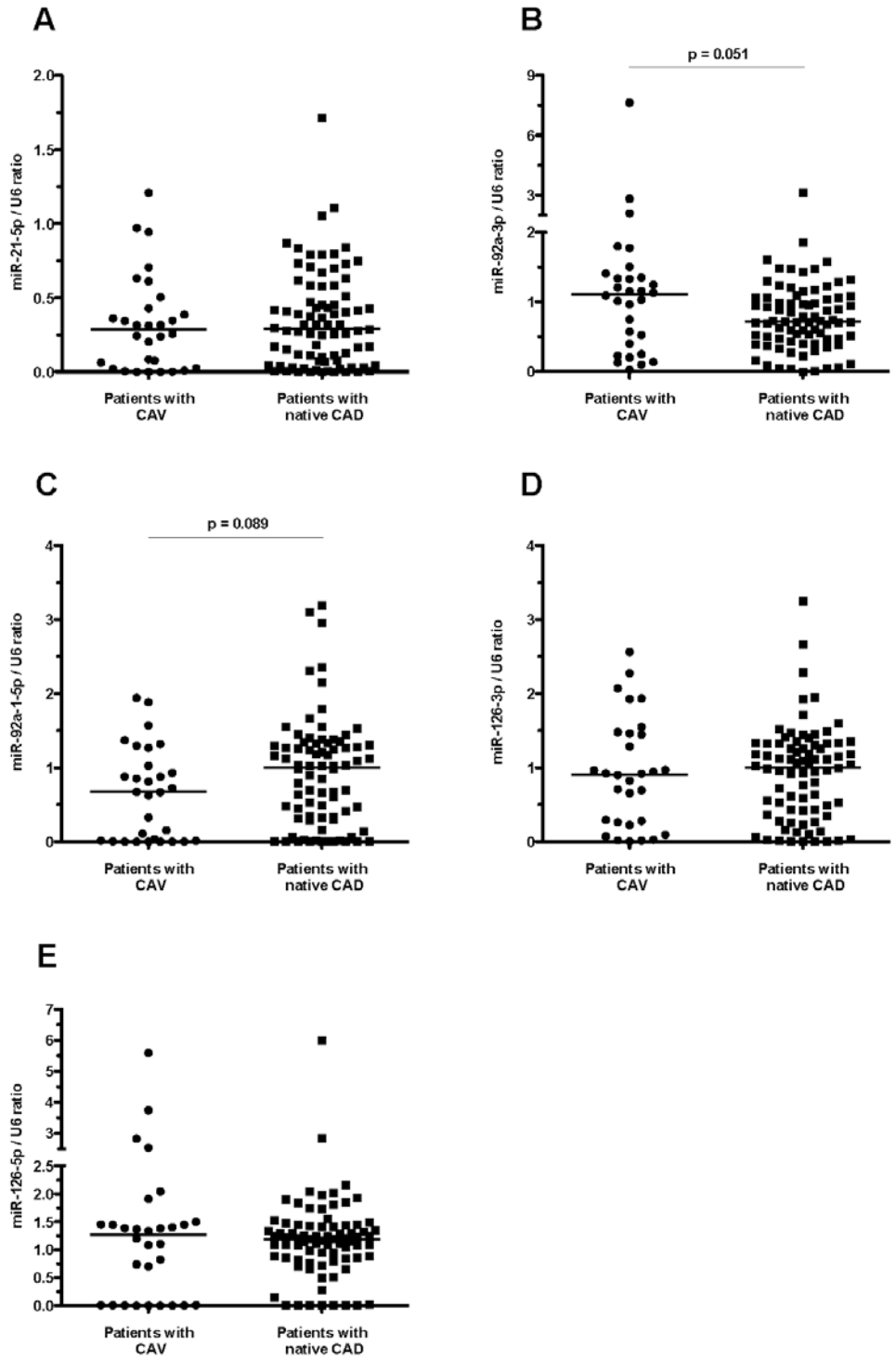

Figure 3.4.2. Individual value bar graph showing a comparison of plasma level of miR-21-5p (panel A), miR-92a-3p (panel B), miR-92a-1-5p (panel C), miR-126-3p (panel D) and miR-126-5p (panel E) in patients with CAV (n=30) versus patients with stable native CAD (n=80). All miRNA levels were normalized against U6 snRNAlevel. Data points show the individual values. Medians are shown by the horizontal lines.

3.4.4.4 Strong correlation between plasma levels of four miRNAs in heart transplant recipients

Table 3.4.2 shows the Spearman's rank correlation matrix of endothelium-enriched miRNAs and apoptotic CECs and apoptotic CEMPs. With the exception of miR-92a-3p, endothelium-enriched miRNAs were strongly correlated (Spearman's rank correlation coefficient higher than 0.8). Plasma levels of miR-21-5p, miR-92a-1-5p, miR-126-3p, and miR-126-5p were weakly correlated with apoptotic CECs whereas the level of miR-92a-3p was weakly correlated with apoptotic CEMPs. Plasma miRNA levels were not related to clinical parameters (data not shown).

3.4.4.5 Logistic regression models for discrimination between CAV-positive and CAV-negative transplant recipients

Table 3.4.3 summarizes the odds ratio per standard deviation increase and the c-statistic values of univariable logistic regression models for clinical and biochemical parameters and endothelial biomarkers. Plasma levels of miR-126-5p and of miR-92a-3p predicted CAV-status significantly better than chance (Table 3.4.4). Since the levels of these two miRNAs were only weakly correlated, miR-126-5p and miR-92a-3p were further analyzed in multivariable logistic regression models. Discrimination between CAV-negative and CAV-positive transplant recipients based on multivariable logistic regression models is shown in Table 3.4.4. Data in relation to the previously published model with age, creatinine, apoptotic CECs, and apoptotic CEMPs as predictors[18] are shown as reference values. The model with age, creatinine, miR-126-5p, and miR-92a-3p as covariables conferred good discrimination between patients without CAV and patients with CAV (c-statistic 0.800 (95% CI 0.674-0.926)). In a logistic regression model with recipient age and creatinine as covariates, miR-126-5p (χ^2=4.37; df=1; p=0.037), miR-92a-3p (χ^2=6.01; df=1; p=0.014), and the combination of miR-126-5p and miR-92a-3p (χ^2=8.162; df=2; p=0.017) added significant information. In addition, miR-92a-3p (χ^2=5.45; df=1; p=0.0195) and not miR-126-5p (chi-square=2.04; df=1; p=0.15) added value in a model with apoptotic CECs and apoptotic CEMPs as predictors. The receiver operating characteristic curve for the logistic regression model with apoptotic CECs, apoptotic CEMPs, and miR-92a-3p as predictors is shown in Figure 3.4.3. Taken together, these data indicate that endothelium-enriched miRNAs have predictive ability for CAV beyond clinical predictors or other endothelial biomarkers.

Table 3.4.2. Spearman's rank correlation matrix of endothelium-enriched miRNAs and apoptotic CECs and apoptotic CEMPs.

	miR-21-5p	miR-92a-3p	miR-92a-1-5p	miR-126-3p	miR-126-5p	Apoptotic CECs	Apoptotic CEMPs
miR-21-5p	1.00	0.552****	0.791****	0.808****	0.754****	0.252	0.176
miR-92a-3p	0.552****	1.00	0.288*	0.479***	0.312*	-0.0612	0.276
miR-92a-1-5p	0.791****	0.288*	1.00	0.855****	0.866****	0.343*	0.0891
miR-126-3p	0.808****	0.479***	0.855****	1.00	0.863****	0.341*	0.180
miR-126-5p	0.754****	0.312*	0.866****	0.863****	1.00	0.351*	0.167
Apoptotic CECs	0.252	-0.0612	0.343*	0.341*	0.351*	1.00	0.0820
Apoptotic CEMPs	0.176	0.276	0.0891	0.180	0.167	0.0820	1.00

CEC: circulating endothelial cell. CEMP: circulating endothelial microparticle. * : p<0.05, ** : p<0.01, *** : p<0.001 and **** : p<0.0001. CECs expessed as ln number/µl. CEMPs expressed as number/µl.

Table 3.4.3. Odds ratios and c-statistic values of univariable logistic regression models for clinical and biochemical parameters and endothelial biomarkers.

	Odds Ratios	P value	c-statistic
Age (years)	1.98 (1.05-3.72)	0.0336	0.689 (0.537-0.842)
Creatinine (mg/dL)	1.99 (1.02-3.86)	0.0425	0.703 (0.552-0.854)
CRP (ln mg/l)	1.74 (0.94-3.21)	0.0776	0.643 (0.488-0.799)
Apoptotic CECs (ln number/µl)	2.32 (1.14-4.71)	0.0196	0.709 (0.567-0.851)
Apoptotic CEMPs (number/µl)	3.24 (1.17-8.96)	0.0234	0.697 (0.554-0.840)
miR-21-5p	1.97 (0.95-4.10)	0.0706	0.617 (0.458-0.776)
miR-92a-3p	4.04 (1.09-14.9)	0.0362	0.682 (0.533-0.831)
miR-92a-1-5p	1.34 (0.75-2.38)	0.327	0.553 (0.394-0.712)
miR-126-3p	1.95 (1.00-3.77)	0.0485	0.647 (0.494-0.800)
miR-126-5p	1.97 (0.91-4.27)	0.0865	0.655 (0.502-0.807)

CEC: circulating endothelial cell. CEMP: circulating endothelial microparticle. Odds ratios are expressed per standard deviation increase. The c-statistic corresponds to the area under the ROC curve. The 95% confidence interval is indicated between brackets.

Table 3.4.4. Discrimination between CAV-negative and CAV-positive transplant recipients based on receiver operating characteristic analysis of multivariable logistic regression models.

	c-statistic (95% CI)
Age + creatinine	0.703 (0.552-0.854)
Apoptotic CECs + apoptotic CEMPs	0.812 (0.692-0.932)
Age + creatinine + apoptotic CECs + apoptotic CEMPs	0.855 (0.756-0.953)
miR-126-5p + miR-92a-3p	0.732 (0.586-0.878)
Age + creatinine + miR-126-5p	0.768 (0.635-0.901)
Age + creatinine + miR-92a-3p	0.783 (0.649-0.917)
Age + creatinine + miR-126-5p + miR-92a-3p	0.800 (0.674-0.926)
Apoptotic CECs + apoptotic CEMPs + miR-126-5p	0.815 (0.699-0.931)
Apoptotic CECs + apoptotic CEMPs + miR-92a-3p	0.847 (0.740-0.954)
Apoptotic CECs + apoptotic CEMPs + miR-126-5p + miR-92a-3p	0.852 (0.746-0.957)

The c-statistic corresponds to the area under the ROC curve. CEC: circulating endothelial cell. CEMP: circulating endothelial microparticle. CI: confidence interval.

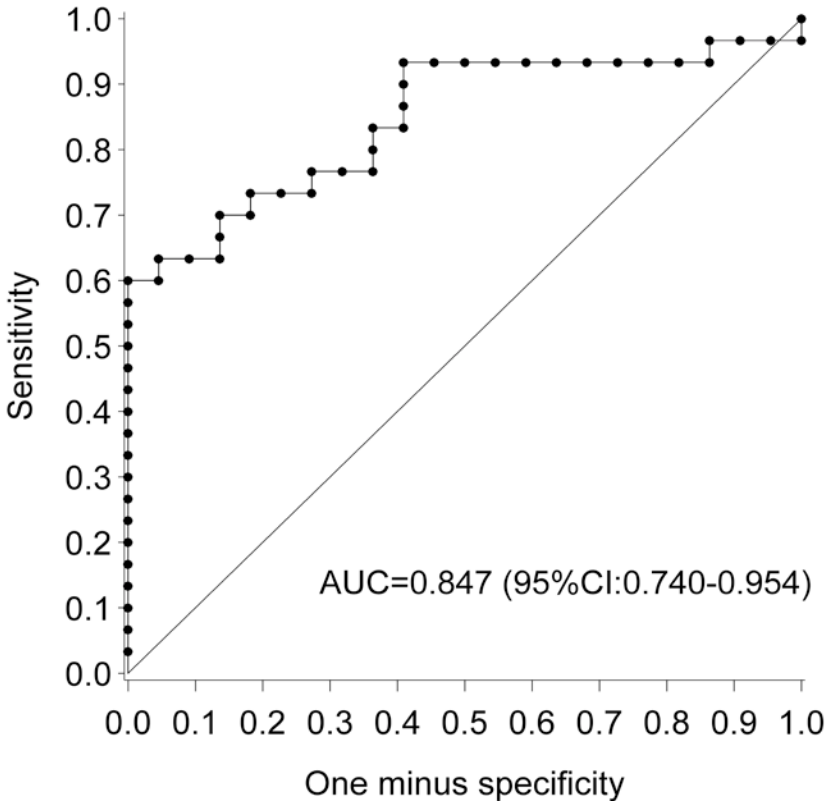

Figure 3.4.3. Receiver-operating characteristic curve for the logistic regression model with apoptotic CECs, apoptotic CEMPs, and miR-92a-3p as predictors. The area under this curve is equal to the C-statistic.

3.4.5 DISCUSSION

The salient findings of the present study are that 1) several endothelium-enriched miRNAs are increased in the plasma of CAV-positive patients compared to CAV-negative patients; 2) whereas all investigated endothelium-enriched miRNAs are increased both in heart transplant recipients with CAV and in patients with native CAD compared to healthy controls, two of five endothelium-enriched miRNAs are distinct in these two types of arteriosclerosis; 3) plasma levels of miR-126-5p and of miR-92a-3p predict CAV-status in univariable models; and 4) these endothelium-enriched miRNAs have predictive ability for CAV beyond clinical predictors or other endothelial biomarkers.

Biomarkers that capture key processes in the pathogenesis of CAV, e.g. biomarkers related to endothelial homeostasis[18], may be the cornerstone for adequate prediction models. The five miRNAs analysed in this study (miR-21-5p, miR-92a-3p, miR-92a-1-5p, miR-126-3p, and miR-126-5p) are endothelium-enriched miRNAs and have been shown to play a role in endothelial homeostasis[21,25]. MiR-21 negatively modulates angiogenesis by targeting RhoB expression[26]. The precursor miRNA miR-21 gives rise to two mature miRNAs: miR-21-3p and miR-21-5p. MiR-21-5p

is an important regulator of neointimal hyperplasia development after balloon injury[27,28]. MiR-92a represses angiogenesis[29, 30] and promotes endothelial activation[31]. MiR-92a-3p and miR-92a-1-5p are two distinct mature miRs produced from the same precursor miRNA pre-miR-92a-1. Finally, miR-126 is one of the most abundant miRNAs in endothelial cells and is involved in the regulation of vascular integrity and angiogenesis[32]. MiR-126-3p and miR-126-5p are two distinct mature miRNAs arising from the same precursor pre-miR-126. MiR-126-3p has been shown to confer anti-inflammatory effects by inhibiting expression of vascular cell adhesion molecule 1 and sprouty-related protein 1[33,34]. MiR-126-5p enhances endothelial proliferation via inhibition of the Notch1 inhibitor delta-like 1 homolog (Dlk1)[35]. Thus, the two miR-126 strands play a role in endothelial repair mechanisms in response to continuous endothelial inflammation and apoptosis resulting in endothelial damage. Taken together, the specific functions of the different miRNAs investigated in the current study may be biologically relevant for the development of CAV. However, the focus of the current study is the development of prediction models of prevalent disease. Therefore, a potential causal role of any of these miRNAs is not under consideration in this report. Interestingly, all investigated miRNAs were increased both in heart transplants recipients with CAV and in patients with native CAD compared to healthy controls. Nevertheless, whereas plasma levels of miR-92a-3p were elevated in CAV-positive patients compared to patients with stable native CAD, the opposite pattern was observed for miR-92a-1-5p. This distinction in endothelial biology between these two types of arteriosclerosis is also reflected by our previous report demonstrating that markers of endothelial injury are distinct in patients with stable native CAD and with CAV[36].

Whereas miRNAs have been investigated as non-invasive biomarkers for heart transplant rejection[37,38], this is the first report to demonstrate the discriminative ability of miRNAs in clinical prediction models of prevalent CAV. A strong correlation of plasma levels of 4 of the 5 investigated endothelium-enriched miRNAs was observed in heart transplant recipients. Therefore, consideration of all miRNAs for multivariable modeling was not meaningful and could have led to multicollinearity problems. Moreover, given the number of patients included in the current study, the number of predictors in multivariable models had to be limited. The model with age, creatinine, miR-126-5p, and miR-92a-3p as covariables conferred good discrimination between patients without CAV and patients with CAV and both endothelium-enriched miRNAs had predictive ability for CAV beyond clinical predictors. However, this model does not provide better discrimination compared to the previously published model with age, creatinine, apoptotic CECs, and apoptotic CEMPs as predictors[18]. Nevertheless, plasma level of miR-92a-3p added value in a model with apoptotic CECs and apoptotic CEMPs as predictors, which may provide a foundation for a model with improved discrimination. Since the number of subjects in the current study was limited to 22 CAV-negative patients and 30 CAV-positive patients, we cannot test whether plasma level of miR-92a-3p adds information beyond the previously established model with age, creatinine, apoptotic CECs, and apoptotic CEMPs as predictors. Inclusion of too many predictors leads to overfitting of the data and C-indices are overestimated[38]. Specifically, models with more than 3 predictors should be interpreted with extreme caution considering the sample size. A larger study is required

to analyse the discriminative ability of a model including clinical predictors, apoptotic CECs, and apoptotic CEMPs, and miR-92a-3p.

There is a biological rational why an endothelium-enriched miRNA has predictive ability for CAV beyond clinical predictors or beyond apoptotic CEMPs and apoptotic CECs. Although miRNAs can be released by apoptosis or necrosis, miRNAs can also enter the circulation in exosomes. Exosomes are built by inward budding of the limiting cell membrane of the multivesicular body, a late endosomal compartment[40]. The fusion of the multivesicular body with the plasma membrane leads to the active secretion of exosomes into the blood circulation. Since this active secretion process is fundamentally distinct from apoptosis or necrosis, it is not surprising that no strong correlation is observed between different miRNAs and apoptotic CECs and apoptotic CEMPs. This lack of a strong correlation is a necessary condition to contribute additional information to the prediction of CAV. Taken together, plasma levels of endothelium-enriched miRNAs reflect at least in part a different dimension of endothelial biology.

In conclusion, the current study enforces the paradigm that endothelial biomarkers constitute a solid foundation for clinical prediction models of CAV. Plasma levels of miR-126-5p and miR-92a-3p added value in models with age and creatinine as predictors. The observation that miR-92a-3p has predictive ability beyond apoptotic CEMPs and apoptotic CECs may lead to the development of models with further improved performance.

3.4.6 REFERENCES

1. Stehlik, J., Edwards, L.B., Kucheryavaya, A.Y., Benden, C., Christie, J.D., Dobbels, F., Kirk, R., Rahmel, A.O. ,Hertz, M.I. The Registry of the International Society for Heart and Lung Transplantation: Twenty-eighth Adult Heart Transplant Report--2011. *J Heart Lung Transplant* **30**, 1078-1094 (2011).
2. Schmauss, D. ,Weis, M. Cardiac allograft vasculopathy: recent developments. *Circulation* **117**, 2131-2141 (2008).
3. Vassalli, G., Gallino, A., Weis, M., von Scheidt, W., Kappenberger, L., von Segesser, L.K. ,Goy, J.J. Alloimmunity and nonimmunologic risk factors in cardiac allograft vasculopathy. *Eur Heart J* **24**, 1180-1188 (2003).
4. Rahmani, M., Cruz, R.P., Granville, D.J. ,McManus, B.M. Allograft vasculopathy versus atherosclerosis. *Circ Res* **99**, 801-815 (2006).
5. Kapadia, S.R., Nissen, S.E. ,Tuzcu, E.M. Impact of intravascular ultrasound in understanding transplant coronary artery disease. *Curr Opin Cardiol* **14**, 140-150 (1999).
6. Ross, R. The pathogenesis of atherosclerosis--an update. *N Engl J Med* **314**, 488-500 (1986).
7. Biadi, O., Potena, L., Fearon, W.F., Luikart, H.I., Yeung, A., Ferrara, R., Hunt, S.A., Mocarski, E.S. ,Valantine, H.A. Interplay between systemic inflammation and markers of insulin resistance in cardiovascular prognosis after heart transplantation. *J Heart Lung Transplant* **26**, 324-330 (2007).
8. Raichlin, E.R., McConnell, J.P., Lerman, A., Kremers, W.K., Edwards, B.S., Kushwaha, S.S., Clavell, A.L., Rodeheffer, R.J. ,Frantz, R.P. Systemic inflammation and metabolic syndrome in cardiac allograft vasculopathy. *J Heart Lung Transplant* **26**, 826-833 (2007).
9. Valantine, H., Rickenbacker, P., Kemna, M., Hunt, S., Chen, Y.D., Reaven, G. ,Stinson, E.B. Metabolic abnormalities characteristic of dysmetabolic syndrome predict the development of transplant coronary artery disease: a prospective study. *Circulation* **103**, 2144-2152 (2001).
10. Nath, D.S., Angaswamy, N., Basha, H.I., Phelan, D., Moazami, N., Ewald, G.A. ,Mohanakumar, T. Donor-specific antibodies to human leukocyte antigens are associated with and precede antibodies to major histocompatibility complex class I-related chain A in antibody-mediated rejection and cardiac allograft vasculopathy after human cardiac transplantation. *Hum Immunol* **71**, 1191-1196 (2010).
11. Acevedo, M.J., Caro-Oleas, J.L., Alvarez-Marquez, A.J., Sobrino, J.M., Lage-Galle, E., Aguilera, I. ,Nunez-Roldan, A. Antibodies against heterogeneous nuclear ribonucleoprotein K in patients with cardiac allograft vasculopathy. *J Heart Lung Transplant* **30**, 1051-1059 (2011).
12. Hognestad, A., Endresen, K., Wergeland, R., Stokke, O., Geiran, O., Holm, T., Simonsen, S., Kjekshus, J.K. ,Andreassen, A.K. Plasma C-reactive protein as a marker of cardiac allograft vasculopathy in heart transplant recipients. *J Am Coll Cardiol* **42**, 477-482 (2003).

13. Singh, N., Jacobs, F., Rader, D.J., Vanhaecke, J., Van Cleemput, J. ,De Geest, B. Impaired cholesterol efflux capacity and vasculoprotective function of high-density lipoprotein in heart transplant recipients. *J Heart Lung Transplant* **33**, 499-506 (2014).
14. Arora, S., Gunther, A., Wennerblom, B., Ueland, T., Andreassen, A.K., Gude, E., Endresen, K., Geiran, O., Wilhelmsen, N., Andersen, R., Aukrust, P. ,Gullestad, L. Systemic markers of inflammation are associated with cardiac allograft vasculopathy and an increased intimal inflammatory component. *Am J Transplant* **10**, 1428-1436 (2010).
15. Arora, S., Andreassen, A., Simonsen, S., Gude, E., Dahl, C., Skaardal, R., Hoel, I., Geiran, O. ,Gullestad, L. Prognostic importance of renal function 1 year after heart transplantation for all-cause and cardiac mortality and development of allograft vasculopathy. *Transplantation* **84**, 149-154 (2007).
16. Schober, A., Hristov, M., Kofler, S., Forbrig, R., Lohr, B., Heussen, N., Zhe, Z., Akhtar, S., Schumann, U., Krotz, F., Leibig, M., Konig, A., Kaczmarek, I., Reichart, B., Klauss, V., Weber, C. ,Sohn, H.Y. CD34+CD140b+ cells and circulating CXCL12 correlate with the angiographically assessed severity of cardiac allograft vasculopathy. *Eur Heart J* **32**, 476-484 (2011).
17. Daly, K.P., Seifert, M.E., Chandraker, A., Zurakowski, D., Nohria, A., Givertz, M.M., Karumanchi, S.A. ,Briscoe, D.M. VEGF-C, VEGF-A and related angiogenesis factors as biomarkers of allograft vasculopathy in cardiac transplant recipients. *J Heart Lung Transplant* **32**, 120-128 (2013).
18. Singh, N., Van Craeyveld, E., Tjwa, M., Ciarka, A., Emmerechts, J., Droogne, W., Gordts, S.C., Carlier, V., Jacobs, F., Fieuws, S., Vanhaecke, J., Van Cleemput, J. ,De Geest, B. Circulating apoptotic endothelial cells and apoptotic endothelial microparticles independently predict the presence of cardiac allograft vasculopathy. *J Am Coll Cardiol* **60**, 324-331 (2012).
19. Suarez, Y., Fernandez-Hernando, C., Yu, J., Gerber, S.A., Harrison, K.D., Pober, J.S., Iruela-Arispe, M.L., Merkenschlager, M. ,Sessa, W.C. Dicer-dependent endothelial microRNAs are necessary for postnatal angiogenesis. *Proc Natl Acad Sci U S A* **105**, 14082-14087 (2008).
20. Kuehbacher, A., Urbich, C., Zeiher, A.M. ,Dimmeler, S. Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. *Circ Res* **101**, 59-68 (2007).
21. Scott, E., Loya, K., Mountford, J., Milligan, G. ,Baker, A.H. MicroRNA regulation of endothelial homeostasis and commitment-implications for vascular regeneration strategies using stem cell therapies. *Free Radic Biol Med* **64**, 52-60 (2013).
22. Yamakuchi, M. MicroRNAs in Vascular Biology. *Int J Vasc Med* **2012**, 794898 (2012).
23. Mehra, M.R., Crespo-Leiro, M.G., Dipchand, A., Ensminger, S.M., Hiemann, N.E., Kobashigawa, J.A., Madsen, J., Parameshwar, J., Starling, R.C. ,Uber, P.A. International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for cardiac allograft vasculopathy-2010. *J Heart Lung Transplant* **29**, 717-727 (2010).

24. Singh, N., Jacobs, F., Rader, D.J., Vanhaecke, J., Van Cleemput, J. ,De Geest, B. Impaired cholesterol efflux capacity and vasculoprotective function of high-density lipoprotein in heart transplant recipients. *J Heart Lung Transplant* **33**, 499-506 (2014).
25. Wang, S. ,Olson, E.N. AngiomiRs--key regulators of angiogenesis. *Curr Opin Genet Dev* **19**, 205-211 (2009).
26. Sabatel, C., Malvaux, L., Bovy, N., Deroanne, C., Lambert, V., Gonzalez, M.L., Colige, A., Rakic, J.M., Noel, A., Martial, J.A. ,Struman, I. MicroRNA-21 exhibits antiangiogenic function by targeting RhoB expression in endothelial cells. *PLoS One* **6**, e16979 (2011).
27. Bauersachs, J. miR-21: a central regulator of fibrosis not only in the broken heart. *Cardiovasc Res* **96**, 227-229; discussion 230-223 (2012).
28. Ji, R., Cheng, Y., Yue, J., Yang, J., Liu, X., Chen, H., Dean, D.B. ,Zhang, C. MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. *Circ Res* **100**, 1579-1588 (2007).
29. Bonauer, A., Carmona, G., Iwasaki, M., Mione, M., Koyanagi, M., Fischer, A., Burchfield, J., Fox, H., Doebele, C., Ohtani, K., Chavakis, E., Potente, M., Tjwa, M., Urbich, C., Zeiher, A.M. ,Dimmeler, S. MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. *Science* **324**, 1710-1713 (2009).
30. Daniel, J.M., Penzkofer, D., Teske, R., Dutzmann, J., Koch, A., Bielenberg, W., Bonauer, A., Boon, R.A., Fischer, A., Bauersachs, J., van Rooij, E., Dimmeler, S. ,Sedding, D.G. Inhibition of miR-92a improves re-endothelialization and prevents neointima formation following vascular injury. *Cardiovasc Res* **103**, 564-572 (2014).
31. Loyer, X., Potteaux, S., Vion, A.C., Guerin, C.L., Boulkroun, S., Rautou, P.E., Ramkhelawon, B., Esposito, B., Dalloz, M., Paul, J.L., Julia, P., Maccario, J., Boulanger, C.M., Mallat, Z. ,Tedgui, A. Inhibition of MicroRNA-92a Prevents Endothelial Dysfunction and Atherosclerosis in Mice. *Circ Res* **114**, 434-443 (2014).
32. Fish, J.E., Santoro, M.M., Morton, S.U., Yu, S., Yeh, R.F., Wythe, J.D., Ivey, K.N., Bruneau, B.G., Stainier, D.Y. ,Srivastava, D. miR-126 regulates angiogenic signaling and vascular integrity. *Dev Cell* **15**, 272-284 (2008).
33. Harris, T.A., Yamakuchi, M., Ferlito, M., Mendell, J.T. ,Lowenstein, C.J. MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. *Proc Natl Acad Sci U S A* **105**, 1516-1521 (2008).
34. Zernecke, A., Bidzhekov, K., Noels, H., Shagdarsuren, E., Gan, L., Denecke, B., Hristov, M., Koppel, T., Jahantigh, M.N., Lutgens, E., Wang, S., Olson, E.N., Schober, A. ,Weber, C. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. *Sci Signal* **2**, ra81 (2009).
35. Schober, A., Nazari-Jahantigh, M., Wei, Y., Bidzhekov, K., Gremse, F., Grommes, J., Megens, R.T., Heyll, K., Noels, H., Hristov, M., Wang, S., Kiessling, F., Olson, E.N. ,Weber, C. MicroRNA-126-5p promotes endothelial proliferation and limits atherosclerosis by suppressing Dlk1. *Nat Med* **20**, 368-376 (2014).

36. Singh, N., Vanhaecke, J., Van Cleemput, J. ,De Geest, B. Markers of endothelial injury and platelet microparticles are distinct in patients with stable native coronary artery disease and with cardiac allograft vasculopathy. *Int J Cardiol* **179**, 331-333 (2015).
37. Sukma Dewi, I., Torngren, K., Gidlof, O., Kornhall, B. ,Ohman, J. Altered serum miRNA profiles during acute rejection after heart transplantation: potential for non-invasive allograft surveillance. *J Heart Lung Transplant* **32**, 463-466 (2013).
38. Duong Van Huyen, J.P., Tible, M., Gay, A., Guillemain, R., Aubert, O., Varnous, S., Iserin, F., Rouvier, P., Francois, A., Vernerey, D., Loyer, X., Leprince, P., Empana, J.P., Bruneval, P., Loupy, A. ,Jouven, X. MicroRNAs as non-invasive biomarkers of heart transplant rejection. *Eur Heart J* **35**, 3194-3202 (2014).
39. Peduzzi, P., Concato, J., Kemper, E., Holford, T.R. ,Feinstein, A.R. A simulation study of the number of events per variable in logistic regression analysis. *J Clin Epidemiol* **49**, 1373-1379 (1996).
40. Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J.J. ,Lotvall, J.O. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* **9**, 654-659 (2007).

Chapter 4

GENERAL DISCUSSION

In order to avoid repetition, the objective of this chapter is not to present a comprehensive discussion of the scientific work of this PhD thesis. The aim of this chapter is to present selected topics that have been not discussed in detail in the different papers comprised in this doctoral thesis.

4.1 C-REACTIVE PROTEIN AS A PREDICTOR OF PREVALENT CAV

As described in chapter 3.3, the median C-reactive protein (CRP) level was 2.24-fold (p=0.082) higher in patients with cardiac allograft vasculopathy (CAV) than in patients without CAV. In a univariable logistic regression model, the odds ratio per standard deviation increase of CRP (ln transformed) was 1.74 (95% CI 0.94-3.21) (p=0.078) and the c-statistic corresponding to this model was 0.643 (95% CI 0.488-0.799). Although statistical significance was not reached, this likely reflects a lack of statistical power. Furthermore, lack of statistical significance in a univariable model does not preclude added value in a multivariable model.

The odds ratios of a multivariable logistic regression model containing apoptotic circulating endothelial cells (CECs) (ln transformed), apoptotic circulating endothelial microparticles (CEMPs), and CRP (ln transformed) as predictors are shown in Table 4.1. CRP (χ^2=9.08; df=1; p=0.0026) added value in the model with apoptotic CECs and apoptotic CEMPs as predictors. The receiver operating characteristic curve of the logistic regression model with apoptotic CECs, apoptotic CEMPs, and CRP as predictors is shown in Figure 4.1. The c-statistic corresponding to this model is 0.870 (95% CI 0.774-0.966).

4.2 OVERFITTING AND OVEROPTIMISM

A key threat to the usefulness of the predictions is overfitting. This refers to the phenomenon that the sample is well described by the model, but that the derived predictions are not valid for new subjects. Overfitting originates from applying a statistical model with too many degrees of freedom (i.e. too many predictors). Degrees of freedom are used (1) in the mere estimation of the coefficients in a regression model ('parameter uncertainty') and (2) in the search of the optimal model structure ('model uncertainty'). This overfitting will lead to overoptimism with regard to the discriminative ability of the model. In a future validation study, a pre-specified model approach can be used instead of a model building approach. In a pre-specified model approach, models with a selected set of *a priori* defined predictors are evaluated. The models that will be considered are the three following:

1. age, creatinine, apoptotic CECs, and apoptotic CEMPs;
2. age, creatinine, apoptotic CECs, and apoptotic CEMPs, CRP;
3. age, creatinine, apoptotic CECs, and apoptotic CEMPs, miR-92a-3p.

Considering that there are 5 predictors in two of these models, a validation study should ideally contain at least 50 CAV negative patients and 50 CAV positive patients[1].

Table 4.1. Logistic regression model containing apoptotic CECs, apoptotic CEMPs and CRP, as predictors for prediction of CAV.

	OR	P value
Apoptotic CECs (ln number/µl)	4.52 (1.52-13.5)	0.0067
Apoptotic CEMPs (number/µl)	6.45 (1.42-29.2)	0.0156
CRP (ln mg/l)	3.73 (1.39-10.0)	0.0090

The 95% confidence interval is indicated between brackets. Odds ratios (OR) are expressed per standard deviation increase.

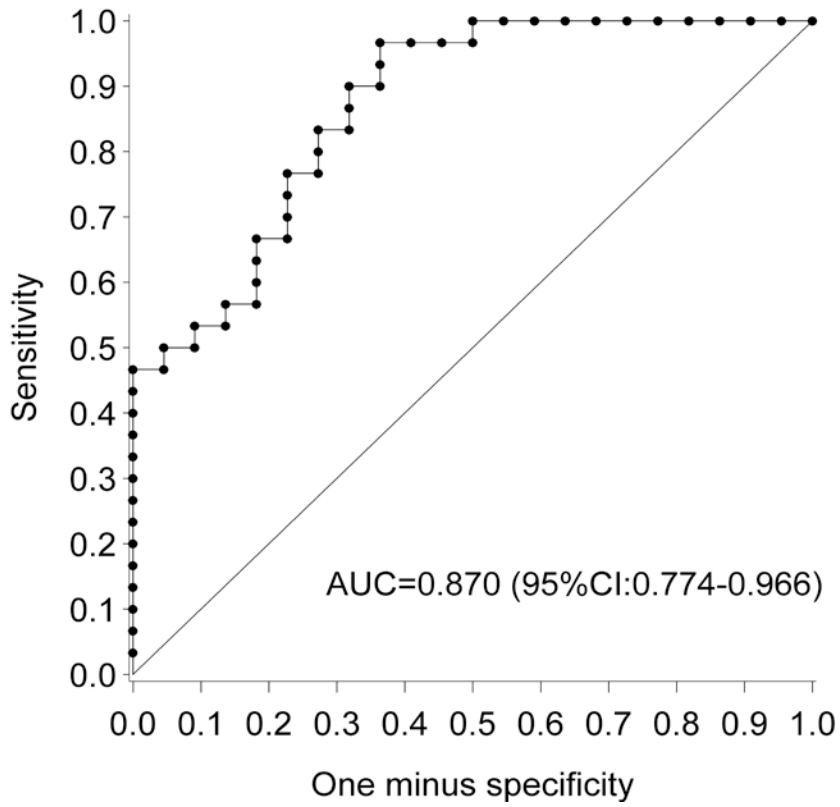

Figure 4.1. Receiver-operating characteristic curve for the logistic regression model with apoptotic CECs, apoptotic CEMPs, and CRP as predictors. The area under this curve is equal to the c-statistic.

4.3 ENDPOINTS IN HEART TRANSPLANTATION RESEARCH AND THE DEVELOPMENT OF PROGNOSTIC PREDICTION MODELS FOR INCIDENT DISEASE

CAV was graded according to the International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for CAV-2010[2]. Therefore, the performance of diagnostic prediction models was evaluated in terms of their ability to predict the prevalence of CAV defined according to angiographic criteria. An angiographic definition of CAV is likely pathophysiologically relevant. However, since intimal proliferation in CAV is circumferential and since the disease process is diffuse extending into intramyocardial vessels, it is not entirely clear how strong the correlation is between angiographic abnormalities and the functional deterioration of the coronary circulation. Furthermore, one should also keep in mind that an angiographic imaging end-point is a surrogate end-point that is used to predict major adverse cardiac events, late graft failure, and mortality.

The development of a prognostic prediction model for incident CAV or hard clinical end-points was not considered. The development of such a model requires a long-term prospective study that is beyond the scope of this doctoral thesis. Intravascular ultrasound (IVUS) is a particularly sensitive method to detect early CAV and may demonstrate significant intimal thickening in the presence of a normal coronary angiogram[3]. The validity of the increase in maximal intimal thickness quantified by IVUS as a surrogate imaging end-point for subsequent angiographic disease and for hard clinical end-points has been demonstrated[3-5]. IVUS could be used to define surrogate imaging end-point in a prospective study. The short-term objective of such a prospective study would be the development of a prediction model (multiple linear regression) for the change (increase) of the maximal intimal thickness (MIT) between 3 weeks and 1 year after transplant. Clearly, the long-term objective would be the prediction of hard clinical end-points. It is clear that such a prospective study would be complicated by a very substantial cost and also by a likely long recruitment phase of patients.

4.4 HDL FUNCTION AND THE HDL HYPOTHESIS

Recently, Rohatgi *et al*.[6] demonstrated that cholesterol efflux capacity, measured at the time of inclusion in 2924 participants in the Dallas Heart Study free from cardiovascular disease at baseline, was inversely associated with the incidence of atherosclerotic cardiovascular disease. This end-point was defined as a first nonfatal myocardial infarction, nonfatal stroke, or coronary revascularization or death from cardiovascular causes. The assay applied by Rohatgi *et al*.[6] is similar to the assay used by Khera *et al*.[7], which is identical to the procedure applied in chapter 3.3 of this doctoral thesis. These assays primarily evaluate cholesterol efflux mediated by ATP-binding cassette transporter A1 (ABCA1). There is one essential difference between the assay of Rohatgi *et al*.[6] and the assay of Khera *et al*.[7]. The fluorescence-labeled reagent boron dipyrromethene difluoride (BODIPY) cholesterol was used by Rohatgi *et al*.[6], whereas efflux was measured using

radiolabeled cholesterol in the study of Khera *et al*.[7]. Surprisingly, the cholesterol efflux capacity evaluated with BODIPY cholesterol was only moderately correlated with measurements performed with radiolabeled cholesterol (correlation coefficient for normalized cholesterol efflux 0.54). This illustrates that these assays are very delicate to execute and that they are not characterized by the same level of reproducibility compared to assays in clinical biology. There is also a complete lack of standardization in this area. *Li et al*.[8] observed that heightened cholesterol efflux to apoB-depleted serum was paradoxically associated with increased prospective risk for myocardial infarction, stroke, and death. This raises the question why these results contrast with those of Rohatgi *et al*.[6]. First of all, due to the specific design of the study of *Li et al*.[8], their findings may reflect index event bias. Secondly, *Li et al*.[8] used THP-1 cells and 2% apo B-depleted serum, whereas in the studies of Khera *et al*. [7] and Rohatgi *et al*.[6] murine J774 cells and 2.8% apo B-depleted serum were used. There are additional distinctions between these assays and all these differences support the thesis that results of these assays have a different significance. Taken together, whereas this field is confounded by a lack of standardization, the recent results of Rohatgi *et al*.[6] support the general validity of the cholesterol efflux assay used in chapter 3.3. However, since this assay was not used in the setting of native atherosclerosis in chapter 3.3 and since this cholesterol efflux capacity is likely highly influenced by the background therapy of these patients, any conclusion beyond the mere fact that cholesterol efflux capacity is substantially reduced in heart transplant recipients is speculative.

According to this HDL hypothesis, raising HDL cholesterol is expected to lead to a decrease of coronary heart disease risk[9]. In a more lipoprotein-centric than cholesterol-centric view, this hypothesis states that raising 'HDL' will lead to a decrease of coronary events. The most important argument against the HDL hypothesis is the recent Mendelian randomization Voight *et al*.[10]. They constructed a genetic score for HDL cholesterol combining the HDL-cholesterol-raising alleles at each of 14 single nucleotide polymorphisms. Each of these single nucleotide polymorphisms had statistical evidence at genome wide levels of significance for association with plasma HDL cholesterol and no evidence for association with triglycerides or LDL cholesterol. In Mendelian randomization analysis, a 1 standard deviation rise in HDL cholesterol due to the genetic score was not associated with risk of myocardial infarction. This is in sharp contrast with lowered risk of myocardial infarction associated with a 1 standard deviation rise in HDL cholesterol in observational epidemiology. Therefore, the possibility that the epidemiological relationship between HDL cholesterol and coronary artery disease reflects residual confounding merits serious consideration.

In the modified HDL hypothesis, it is postulated that enhanced HDL function will result in a reduction of coronary events[9]. However, the shift from a biochemical surrogate end-point to a functional surrogate end-point does not solve by itself the uncertain value of surrogate end-points[9]. Taken together, the HDL hypothesis remains unproven. The study of HDL function in heart transplant recipients is unlikely to yield substantial additional information beyond the results described in chapter 3.3.

4.5 REFERENCES

1. Peduzzi, P., Concato, J., Kemper, E., Holford, T.R. ,Feinstein, A.R. A simulation study of the number of events per variable in logistic regression analysis. *J Clin Epidemiol* **49**, 1373-1379 (1996).
2. Mehra, M.R., Crespo-Leiro, M.G., Dipchand, A., Ensminger, S.M., Hiemann, N.E., Kobashigawa, J.A., Madsen, J., Parameshwar, J., Starling, R.C. ,Uber, P.A. International Society for Heart and Lung Transplantation working formulation of a standardized nomenclature for cardiac allograft vasculopathy-2010. *J Heart Lung Transplant* **29**, 717-727 (2010).
3. Kapadia, S.R., Nissen, S.E. ,Tuzcu, E.M. Impact of intravascular ultrasound in understanding transplant coronary artery disease. *Curr Opin Cardiol* **14**, 140-150 (1999).
4. Kobashigawa, J.A., Tobis, J.M., Starling, R.C., Tuzcu, E.M., Smith, A.L., Valantine, H.A., Yeung, A.C., Mehra, M.R., Anzai, H., Oeser, B.T., Abeywickrama, K.H., Murphy, J. ,Cretin, N. Multicenter intravascular ultrasound validation study among heart transplant recipients: outcomes after five years. *J Am Coll Cardiol* **45**, 1532-1537 (2005).
5. Tuzcu, E.M., Kapadia, S.R., Sachar, R., Ziada, K.M., Crowe, T.D., Feng, J., Magyar, W.A., Hobbs, R.E., Starling, R.C., Young, J.B., McCarthy, P. ,Nissen, S.E. Intravascular ultrasound evidence of angiographically silent progression in coronary atherosclerosis predicts long-term morbidity and mortality after cardiac transplantation. *J Am Coll Cardiol* **45**, 1538-1542 (2005).
6. Rohatgi, A., Khera, A., Berry, J.D., Givens, E.G., Ayers, C.R., Wedin, K.E., Neeland, I.J., Yuhanna, I.S., Rader, D.R., de Lemos, J.A. ,Shaul, P.W. HDL cholesterol efflux capacity and incident cardiovascular events. *N Engl J Med* **371**, 2383-2393 (2014).
7. Khera, A.V., Cuchel, M., de la Llera-Moya, M., Rodrigues, A., Burke, M.F., Jafri, K., French, B.C., Phillips, J.A., Mucksavage, M.L., Wilensky, R.L., Mohler, E.R., Rothblat, G.H. ,Rader, D.J. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *N Engl J Med* **364**, 127-135 (2011).
8. Li, X.M., Tang, W.H., Mosior, M.K., Huang, Y., Wu, Y., Matter, W., Gao, V., Schmitt, D., Didonato, J.A., Fisher, E.A., Smith, J.D. ,Hazen, S.L. Paradoxical association of enhanced cholesterol efflux with increased incident cardiovascular risks. *Arterioscler Thromb Vasc Biol* **33**, 1696-1705 (2013).
9. Gordts, S.C., Singh, N., Muthuramu, I. ,De Geest, B. Pleiotropic effects of HDL: towards new therapeutic areas for HDL-targeted interventions. *Curr Mol Med* **14**, 481-503 (2014).
10. Voight, B.F., Peloso, G.M., Orho-Melander, M., Frikke-Schmidt, R., Barbalic, M., Jensen, M.K., Hindy, G., Holm, H., Ding, E.L., Johnson, T., Schunkert, H., Samani, N.J., Clarke, R., Hopewell, J.C., Thompson, J.F., Li, M., Thorleifsson, G., Newton-Cheh, C., Musunuru, K., Pirruccello, J.P., Saleheen, D., Chen, L., Stewart, A., Schillert, A., Thorsteinsdottir, U., Thorgeirsson, G., Anand, S., Engert, J.C., Morgan, T., Spertus, J., Stoll, M., Berger, K.,

Martinelli, N., Girelli, D., McKeown, P.P., Patterson, C.C., Epstein, S.E., Devaney, J., Burnett, M.S., Mooser, V., Ripatti, S., Surakka, I., Nieminen, M.S., Sinisalo, J., Lokki, M.L., Perola, M., Havulinna, A., de Faire, U., Gigante, B., Ingelsson, E., Zeller, T., Wild, P., de Bakker, P.I., Klungel, O.H., Maitland-van der Zee, A.H., Peters, B.J., de Boer, A., Grobbee, D.E., Kamphuisen, P.W., Deneer, V.H., Elbers, C.C., Onland-Moret, N.C., Hofker, M.H., Wijmenga, C., Verschuren, W.M., Boer, J.M., van der Schouw, Y.T., Rasheed, A., Frossard, P., Demissie, S., Willer, C., Do, R., Ordovas, J.M., Abecasis, G.R., Boehnke, M., Mohlke, K.L., Daly, M.J., Guiducci, C., Burtt, N.P., Surti, A., Gonzalez, E., Purcell, S., Gabriel, S., Marrugat, J., Peden, J., Erdmann, J., Diemert, P., Willenborg, C., Konig, I.R., Fischer, M., Hengstenberg, C., Ziegler, A., Buysschaert, I., Lambrechts, D., Van de Werf, F., Fox, K.A., El Mokhtari, N.E., Rubin, D., Schrezenmeir, J., Schreiber, S., Schafer, A., Danesh, J., Blankenberg, S., Roberts, R., McPherson, R., Watkins, H., Hall, A.S., Overvad, K., Rimm, E., Boerwinkle, E., Tybjaerg-Hansen, A., Cupples, L.A., Reilly, M.P., Melander, O., Mannucci, P.M., Ardissino, D., Siscovick, D., Elosua, R., Stefansson, K., O'Donnell, C.J., Salomaa, V., Rader, D.J., Peltonen, L., Schwartz, S.M., Altshuler, D. ,Kathiresan, S. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. *Lancet* **380**, 572-580 (2012).

Summary

Cardiac allograft vasculopathy (CAV) is a limiting factor for the long-term survival of heart transplant recipients[1,2]. CAV is characterized by the development of diffuse concentric fibromuscular intimal hyperplasia in epicardial and smaller intramyocardial arteries along with focal, eccentric atherosclerotic plaques in the larger epicardial arteries[3,4]. The development of these lesions may lead to the progressive narrowing of the lumen[5]. According to the response to injury hypothesis of CAV, these lesions are the result of cumulative endothelial injury induced by alloimmune responses as well as non-immunological risk factors such as ischemia-reperfusion injury, viral infections, and metabolic disorders[3,6].

Early diagnosis of CAV is essential to implement appropriate prevention and treatment measures. Clinical prediction models of CAV are currently not available and may be useful for non-invasive diagnostic and prognostic purposes. The general aim of this doctoral thesis is to develop diagnostic prediction models for prevalent CAV. The specific central hypothesis of this doctoral thesis is that biomarkers of endothelial homeostasis discriminate between CAV-negative and CAV-positive heart transplant recipients.

Endothelial homeostasis reflects the balance between endothelial injury and endothelial repair. In chapter 3.1, we investigated whether biomarkers related to endothelial injury and endothelial repair discriminate between CAV-negative and CAV-positive heart transplant recipients. Fifty-two patients undergoing coronary angiography between 5 and 15 years after heart transplantation were recruited in this study. Flow cytometry was applied to quantify endothelial progenitor cells (EPCs), circulating endothelial cells (CECs), and circulating endothelial microparticles (CEMPs). Cell culture was used for quantification of circulating EPC number and hematopoietic progenitor cell (HPC) number and for analysis of EPC function. EPC number and EPC function did not differ between CAV-negative and CAV-positive patients. In univariable models, age, creatinine, steroid dose, granulocyte colony-forming units, apoptotic CECs, and apoptotic CEMPs discriminated between CAV-positive and CAV-negative patients. The logistic regression model containing apoptotic CECs and apoptotic CEMPs as independent predictors provided high discrimination between CAV-positive and CAV-negative patients (c-statistic 0.812; 95% CI 0.692-0.932). In a

logistic regression model with age and creatinine as covariates, apoptotic CECs (p=0.0112) and apoptotic CEMPs (p=0.0141) were independent predictors (c-statistic 0.855; 95% CI 0.756-0.953). These two biomarkers remained independent predictors when steroid dose was introduced in the model. Taken together, the high discriminative ability of apoptotic CECs and apoptotic CEMPs is a solid foundation for the development of clinical prediction models of CAV.

In chapter 3.2, patients with stable native coronary artery disease (CAD) were compared with heart transplant recipients with CAV. After all, CAV is a particular type of arteriosclerosis with many similarities but also significant differences compared to native CAD. Atherosclerosis in patients with stable native CAD is characterized by the presence of atheromata that contain a lipid core filled with extracellular cholesterol and cellular debris and are covered by a fibrous cap. In contrast, fibromuscular intimal hyperplasia is the most prominent lesion type of CAV and mainly consists of smooth muscle cells and extracellular matrix[7]. Endothelial injury is assumed to play a key role in the initiation and progression of both native CAD and CAV[2,8]. In the response-to-injury hypothesis of atherosclerosis of Ross and Glomset, endothelial injury was originally defined as endothelial denudation resulting from focal desquamation of endothelium[9,10]. Later versions of the response-to-injury hypothesis emphasized endothelial dysfunction rather than denudation[8,11]. Cellular biomarkers of endothelial injury (CEMPs and CECs) may discriminate between endothelial activation and irreversible endothelial damage. The hypothesis that endothelial injury and circulating platelet microparticles (CPMPs) are distinct in both types of arteriosclerosis was investigated.

The geometric mean of the concentration of CECs ($CD45^-$ $CD31^{bright}$ $VEGFR\text{-}2^+$) was 2.90-fold ($p<0.001$) and 2.34-fold ($p<0.05$) higher in patients with stable native CAD (n=80) and with CAV (n=30), respectively, compared to healthy controls (n=25). No significant difference in total, Annexin V negative, and Annexin V positive (apoptotic) CECs was observed between patients with native CAD and with CAV. The concentration of Annexin V negative CEMPs ($CD144^+$ $CD42a^-$) was 59.2% ($p<0.01$) higher in transplant recipients with CAV than in native CAD patients but no difference in Annexin V positive CEMPs was observed. The median value of total $CD61^+$ CPMPs in native CAD patients was 69.4% ($p<0.001$) and 71.6% ($p<0.001$) lower compared to healthy controls and transplant recipients with CAV, respectively. These differences were even more pronounced when $CD42a^+CD31^+$ CPMPs were quantified. In conclusion, the selective increase of Annexin V negative CEMPs and the absence of a difference in Annexin V positive CECs strongly suggest increased endothelial activation but not endothelial apoptosis in CAV-positive patients compared to stable CAD patients. Use of antiplatelet drugs likely underlies the strikingly lower levels of CPMPs in patients with native CAD.

In chapter 3.3, the relation between high density lipoproteins (HDL) and CAV was investigated. The prevalence and the incidence of CAV have been reported to be increased in heart transplant recipients with decreased high density lipoprotein (HDL) cholesterol levels[12-15]. The association between HDL cholesterol and CAV may reflect causation but might also be due to residual confounding. One such confounding factor is insulin resistance, which is considered to play a role in the pathogenesis of CAV. A triglyceride/HDL cholesterol ratio of greater than 3 has been

recognized as a marker of insulin resistance in overweight subjects[16] and constituted a risk factor for CAV and major adverse cardiac events in heart transplant recipients[17,18].

Remodelling of HDL in heart transplant recipients is significantly affected by a lower activity of cholesterol ester transfer protein, phospholipid transfer protein, and hepatic lipase[19,20]. Consequently, these patients are characterized by an increased proportion of large HDL particles and reduced pre-ß1-HDL in the presence of normal or even elevated HDL cholesterol levels[19,20]. These alterations may be partially explained by corticosteroid use[21] but may also be potentiated by statin intake[22]. The modified HDL metabolism and associated compositional changes of HDL particles may lead to an impaired function of these lipoproteins. Reduced HDL function may also occur as a result of ongoing inflammation[23].

We hypothesized that HDL function may be impaired in these patients and may discriminate between CAV-positive and CAV-negative patients. Cholesterol efflux capacity of apolipoprotein B-depleted plasma was analysed using a validated assay[24]. The vasculoprotective function of HDL was studied by means of an EPC migration assay. HDL cholesterol levels were similar in heart transplant patients compared to healthy controls. However, normalized cholesterol efflux and vasculoprotective function were reduced by 24.1% ($p<0.001$) and by 27.0% ($p<0.01$), respectively, in heart transplant recipients compared to healthy controls. HDL function was similar in patients with and without cardiac allograft vasculopathy (CAV) and was not related to C-reactive protein (CRP) levels. An interaction effect ($p=0.0584$) was observed between etiology of heart failure before transplantation and steroid use as factors of HDL cholesterol levels. Lower HDL cholesterol levels occurred in patients with prior ischemic cardiomyopathy not taking steroids. However, HDL function was independent of the etiology of heart failure before transplantation and steroid use. The median C-reactive protein (CRP) level was 2.24-fold ($p=0.082$) higher in patients with CAV than in patients without CAV. In conclusion, HDL function is impaired in heart transplant recipients but is unrelated to CAV-status. The proportion of patients with a CRP level greater than or equal to 6 mg/l is prominently higher in CAV-positive patients.

In chapter 3.4, the potential of endothelium-enriched microRNAs (miRNAs) as putative biomarkers for the prediction of CAV was investigated. MiRNAs are small, non-coding, single-stranded RNA sequences that regulate gene expression at the post-transcriptional level. Because miRNAs circulate in remarkably stable forms in blood[25,26], they have a significant potential as biomarkers. Several reports indicate that miRNAs may play a role in endothelial homeostasis[27,28]. In this study, a candidate-based approach using circulating levels of endothelium-enriched miRNAs (miR-21-5p, miR-92a-3p, miR-92a-1-5p, miR-126-3p, miR-126-5p) to predict CAV was evaluated. Circulating levels of endothelium-enriched miRNAs (miR-21-5p, miR-92a-3p, miR-92a-1-5p, miR-126-3p, miR-126-5p) were quantified by real-time RT-PCR. The discriminative ability of logistic regression models was quantified using the concordance statistic (c-statistic). Plasma levels of miR-21-5p, miR-92a-3p, miR-126-3p, and miR-126-5p were 1.86-fold (p=NS), 1.91-fold ($p<0.05$), 1.74-fold ($p=0.074$), and 1.73-fold ($p=0.060$) higher, in patients with CAV than in patients without CAV. Recipient age (c-statistic 0.689 (95% CI 0.537-0.842)), serum creatinine (c-statistic 0.703 (95% CI 0.552-0.854)), levels of miR-92a-3p (c-statistic 0.682 (95% CI 0.533-0.831)), and levels of miR-

126-5p (c-statistic 0.655 (95% CI 0.502-0.807)) predicted CAV-status in univariable models. In a multivariable logistic regression model with recipient age and creatinine as covariates, miR-126-5p (chi-square=4.374; df=1; p=0.0365), miR-92a-3p (chi-square=6.007; df=1; p=0.0143), and the combination of miR-126-5p and miR-92a-3p (chi square=8.162; df=2; p=0.0169) added significant information. The model with age, creatinine, miR-126-5p and miR-92a-3p as covariables conferred good discrimination between patients without CAV and patients with CAV (c-statistic 0.800 (95% CI 0.674-0.926)). In addition, miR-92a-3p (chi-square=5.454; df=1; p=0.0195) and not miR-126-5p (chi-square=2.037; df=1; p=0.1535) added value in a model with apoptotic CECs and apoptotic CEMPs as predictors (c-statistic0.847 (95% CI 0.740-0.954)). In conclusion, endothelium-enriched miRNAs have predictive ability for CAV beyond clinical predictors.

The central hypothesis at the start of this doctoral thesis was that biomarkers of endothelial homeostasis discriminate between CAV-negative and CAV-positive heart transplant recipients. The validity of this hypothesis has been convincingly demonstrated. The refinement and validation of these models in a larger follow-up study may lead to a clinically useful model that can be applied for monitoring heart transplant recipients.

REFERENCES

1. Stehlik, J., Edwards, L.B., Kucheryavaya, A.Y., Benden, C., Christie, J.D., Dobbels, F., Kirk, R., Rahmel, A.O. ,Hertz, M.I. The Registry of the International Society for Heart and Lung Transplantation: Twenty-eighth Adult Heart Transplant Report--2011. *J Heart Lung Transplant* **30**, 1078-1094 (2011).
2. Schmauss, D. ,Weis, M. Cardiac allograft vasculopathy: recent developments. *Circulation* **117**, 2131-2141 (2008).
3. Vassalli, G., Gallino, A., Weis, M., von Scheidt, W., Kappenberger, L., von Segesser, L.K. ,Goy, J.J. Alloimmunity and nonimmunologic risk factors in cardiac allograft vasculopathy. *Eur Heart J* **24**, 1180-1188 (2003).
4. Rahmani, M., Cruz, R.P., Granville, D.J. ,McManus, B.M. Allograft vasculopathy versus atherosclerosis. *Circ Res* **99**, 801-815 (2006).
5. Kapadia, S.R., Nissen, S.E. ,Tuzcu, E.M. Impact of intravascular ultrasound in understanding transplant coronary artery disease. *Curr Opin Cardiol* **14**, 140-150 (1999).
6. Ross, R. The pathogenesis of atherosclerosis--an update. *N Engl J Med* **314**, 488-500 (1986).
7. Lu, W.H., Palatnik, K., Fishbein, G.A., Lai, C., Levi, D.S., Perens, G., Alejos, J., Kobashigawa, J. ,Fishbein, M.C. Diverse morphologic manifestations of cardiac allograft vasculopathy: a pathologic study of 64 allograft hearts. *J Heart Lung Transplant* **30**, 1044-1050 (2011).
8. Ross, R. Atherosclerosis--an inflammatory disease. *N Engl J Med* **340**, 115-126. (1999).
9. Ross, R. ,Glomset, J.A. Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. *Science* **180**, 1332-1339 (1973).
10. Ross, R. ,Glomset, J.A. The pathogenesis of atherosclerosis (first of two parts). *N Engl J Med* **295**, 369-377 (1976).
11. Ross, R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* **362**, 801-809. (1993).
12. Parameshwar, J., Foote, J., Sharples, L., Wallwork, J., Large, S. ,Schofield, P. Lipids, lipoprotein (a) and coronary artery disease in patients following cardiac transplantation. *Transpl Int* **9**, 481-485 (1996).
13. Valantine, H., Rickenbacker, P., Kemna, M., Hunt, S., Chen, Y.D., Reaven, G. ,Stinson, E.B. Metabolic abnormalities characteristic of dysmetabolic syndrome predict the development of transplant coronary artery disease: a prospective study. *Circulation* **103**, 2144-2152 (2001).
14. Cooke, G.E., Eaton, G.M., Whitby, G., Kennedy, R.A., Binkley, P.F., Moeschberger, M.L. ,Leier, C.V. Plasma atherogenic markers in congestive heart failure and posttransplant (heart) patients. *J Am Coll Cardiol* **36**, 509-516 (2000).

15. Sanchez-Gomez, J.M., Martinez-Dolz, L., Sanchez-Lazaro, I., Almenar, L., Sanchez-Lacuesta, E., Munoz-Giner, B., Portoles, M., Rivera, M., Valera-Roman, A., Gonzalez-Juanatey, J.R., Tejada-Ponce, D., Aguero, J., Buendia, F. ,Salvador, A. Influence of metabolic syndrome on development of cardiac allograft vasculopathy in the transplanted heart. *Transplantation* **93**, 106-111 (2012).
16. McLaughlin, T., Abbasi, F., Cheal, K., Chu, J., Lamendola, C. ,Reaven, G. Use of metabolic markers to identify overweight individuals who are insulin resistant. *Ann Intern Med* **139**, 802-809 (2003).
17. Biadi, O., Potena, L., Fearon, W.F., Luikart, H.I., Yeung, A., Ferrara, R., Hunt, S.A., Mocarski, E.S. ,Valantine, H.A. Interplay between systemic inflammation and markers of insulin resistance in cardiovascular prognosis after heart transplantation. *J Heart Lung Transplant* **26**, 324-330 (2007).
18. Raichlin, E.R., McConnell, J.P., Lerman, A., Kremers, W.K., Edwards, B.S., Kushwaha, S.S., Clavell, A.L., Rodeheffer, R.J. ,Frantz, R.P. Systemic inflammation and metabolic syndrome in cardiac allograft vasculopathy. *J Heart Lung Transplant* **26**, 826-833 (2007).
19. Atger, V., Leclerc, T., Cambillau, M., Guillemain, R., Marti, C., Moatti, N. ,Girard, A. Elevated high density lipoprotein concentrations in heart transplant recipients are related to impaired plasma cholesteryl ester transfer and hepatic lipase activity. *Atherosclerosis* **103**, 29-41 (1993).
20. Sviridov, D., Chin-Dusting, J., Nestel, P., Kingwell, B., Hoang, A., Olchawa, B., Starr, J. ,Dart, A. Elevated HDL cholesterol is functionally ineffective in cardiac transplant recipients: evidence for impaired reverse cholesterol transport. *Transplantation* **81**, 361-366 (2006).
21. Stamler, J.S., Vaughan, D.E. ,Loscalzo, J. Immunosuppressive therapy and lipoprotein abnormalities after cardiac transplantation. *Am J Cardiol* **68**, 389-391 (1991).
22. Yamashita, S., Tsubakio-Yamamoto, K., Ohama, T., Nakagawa-Toyama, Y. ,Nishida, M. Molecular mechanisms of HDL-cholesterol elevation by statins and its effects on HDL functions. *J Atheroscler Thromb* **17**, 436-451 (2010).
23. Fisher, E.A., Feig, J.E., Hewing, B., Hazen, S.L. ,Smith, J.D. High-density lipoprotein function, dysfunction, and reverse cholesterol transport. *Arterioscler Thromb Vasc Biol* **32**, 2813-2820 (2012).
24. Khera, A.V., Cuchel, M., de la Llera-Moya, M., Rodrigues, A., Burke, M.F., Jafri, K., French, B.C., Phillips, J.A., Mucksavage, M.L., Wilensky, R.L., Mohler, E.R., Rothblat, G.H. ,Rader, D.J. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *N Engl J Med* **364**, 127-135 (2011).
25. Suarez, Y., Fernandez-Hernando, C., Yu, J., Gerber, S.A., Harrison, K.D., Pober, J.S., Iruela-Arispe, M.L., Merkenschlager, M. ,Sessa, W.C. Dicer-dependent endothelial microRNAs are necessary for postnatal angiogenesis. *Proc Natl Acad Sci U S A* **105**, 14082-14087 (2008).

26. Kuehbacher, A., Urbich, C., Zeiher, A.M. ,Dimmeler, S. Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. *Circ Res* **101**, 59-68 (2007).
27. Scott, E., Loya, K., Mountford, J., Milligan, G. ,Baker, A.H. MicroRNA regulation of endothelial homeostasis and commitment-implications for vascular regeneration strategies using stem cell therapies. *Free Radic Biol Med* **64**, 52-60 (2013).
28. Yamakuchi, M. MicroRNAs in Vascular Biology. *Int J Vasc Med* **2012**, 794898 (2012).

Curriculum vitae

Name	Neha Singh
Email	neha.singh@med.kuleuven.be
Work phone	+32-(0) 16372087

Education and professional career

2007	Bachelor of Science (Biotechnology) M. J. P. Rohilkhand University, Bareilly, India
2009	Master of Science (Biochemistry) Jiwaji University, Gwalior, India
Master Thesis	Studies on chelation of iron by glycine rich peptides Promoter: Dr. Uma V. Manjapparra Central Food Technological Research Institute (CFTRI), Mysore, India
2010	Awarded with Godawati Satyanarayan Gold Medal
2010-2012	Pre-Doctoral programme Promoter: Prof. Dr. Bart De Geest Co-promoter: Prof. Dr. Johan Van Cleemput Center for Molecular and Vascular Biology Doctoral School Biomedical Sciences Campus Gasthuisberg Catholic University of Leuven, Belgium
2012-Present	PhD student at the Catholic university of Leuven Promoter: Professor Bart De Geest Co-promoter: Prof. Dr. Johan Van Cleemput Center for Molecular and Vascular Biology Doctoral School Biomedical Sciences Campus Gasthuisberg Catholic University of Leuven, Belgium

Publications in international peer reviewed journals

1. **Singh, N.**, Heggermont, W., Fieuws, S., Vanhaecke, J., Van Cleemput, J., De Geest, B. (2014). Endothelium-enriched microRNAs predict the presence of cardiac allograft vasculopathy. Submitted.

2. **Singh, N.**, Vanhaecke, J., Van Cleemput, J., De Geest, B. (2015). Markers of endothelial injury and platelet microparticles are distinct in patients with stable native coronary artery disease and with cardiac allograft vasculopathy. International Journal of Cardiology, 179, 331-333.

3. Van Linthout, S., Frias, M., **Singh, N.**, De Geest, B. (2014). Therapeutic potential of HDL in cardioprotection and tissue repair. Handbook of Experimental Pharmacology, In press

4. **Singh, N.**, Jacobs, F., Rader, D., Vanhaecke, J., Van Cleemput, J., De Geest, B. (2014). Impaired cholesterol efflux capacity and vasculoprotective function of HDL in heart transplant recipients. Journal of Heart and Lung Transplantation, 33 (5), 499-506.

5. Muthuramu, I., Jacobs, F., **Singh, N.**, Gordts, S., De Geest, B. (2013). Selective homocysteine lowering gene transfer improves infarct healing, attenuates remodelling, and enhances diastolic function after myocardial infarction in mice. PLoS One, 8 (5), e63710.

6. Gordts, S., **Singh, N.**, Muthuramu, I., De Geest, B. (2013). Pleiotropic effects of HDL: towards new therapeutic areas for HDL-targeted interventions. Current Molecular Medicine , 14, 1-23.

7. Gordts, S., Van Craeyveld, E., Muthuramu, I., **Singh, N.**, Jacobs, F., De Geest, B. (2012). Lipid Lowering and HDL Raising Gene Transfer Increase Endothelial Progenitor Cells, Enhance Myocardial Vascularity, and Improve Diastolic Function. PLoS One, 7(10), e46849.

8. **Singh, N.**, Van Craeyveld, E., Tjwa, M., Ciarka, A., Emmerechts, J., Droogne, W., Gordts, S., Carlier, V., Jacobs, F., Fieuws, S., Vanhaecke, J., Van Cleemput, J., De Geest, B. (2012). Circulating apoptotic endothelial cells and apoptotic endothelial microparticles independently predict the presence of cardiac allograft vasculopathy. Journal of the American College of Cardiology, 60(4): 324-331.

9. Van Craeyveld, E., Gordts, S., **Singh, N.**, Jacobs, F., De Geest, B. (2012). A critical reassessment of murine and rabbit models of atherosclerosis: focus on lesion progression and remodeling. Acta Cardiologica, 67 (1), 11-21.

Abstracts at international meetings

1. **Singh, N**., Van Craeyveld, E., Tjwa, M., Ciarka, A., Droogne, W., Gordts, S., Jacobs, F., Vanhaecke, J., Van Cleemput, J., De Geest, B. (2012). Circulating apoptotic endothelial cells and apoptotic endothelial microparticles independently predict the presence of cardiac allograft vasculopathy. European Heart Journal: vol. 33. European Society of Cardiology Congress. Munich, Germany, 25-29 August 2012, 752-752.

2. **Singh, N.**, Jacobs, F., Rader, D., Vanhaecke, J., Van Cleemput, J., De Geest, B. (2014). Impaired cholesterol efflux capacity and vasculoprotective function of HDL in heart transplant recipients. 7th IAS Workshop on HDL 2014. Rome, March 26-28 2014.